

=> d his; log hold

(FILE 'HOME' ENTERED AT 12:20:22 ON 04 JUN 2001)

FILE 'MEDLINE' ENTERED AT 12:20:38 ON 04 JUN 2001

E COMPLEMENT/CT
E E3+ALL/CT
E E36+ALL
L1 3009 S (C 1Q) OR (C1 Q) OR C1Q OR (COMPLEMENT(A) (C1Q OR 1Q))
L2 226 S ((C1Q OR COLLECTIN OR L1) (2A) RECEPTOR#)
E CUB/CT
- E CUB DOMAIN/CT
L3 54 S (CUB OR (COMPLEMENT UBIQUITIN)) (2A) (DOMAIN# OR MODULE# OR REG
L4 1 S L2 AND L3
L5 2 S L1 AND L3
L6 1 S L5 NOT L4

FILE 'STNGUIDE' ENTERED AT 12:34:22 ON 04 JUN 2001

FILE 'MEDLINE' ENTERED AT 12:38:07 ON 04 JUN 2001

E E63+ALL
L7 5197 S (INHIBIT? OR ANTAGON? OR INACTIVAT?) (3A) COMPLEMENT###
L8 217 S (INHIBIT? OR ANTAGON? OR INACTIVAT?) (3A) L1
L9 12444 S L2 OR COLLECTIN#
L10 25 S L9 AND L7
L11 42 S L9 AND L8
L12 34 S L11 NOT L10

FILE 'STNGUIDE' ENTERED AT 12:43:38 ON 04 JUN 2001

L13 1 S ISCHEMI? OR INFARCT? OR ARTHRITIS OR LUPUS OR NEPHRITIS OR AL

FILE 'MEDLINE' ENTERED AT 13:00:54 ON 04 JUN 2001

L14 404842 S ISCHEMI? OR INFARCT? OR ARTHRITIS OR LUPUS OR NEPHRITIS OR AL
L15 37 S L14 AND L8

FILE 'STNGUIDE' ENTERED AT 13:02:51 ON 04 JUN 2001

FILE 'MEDLINE' ENTERED AT 13:02:57 ON 04 JUN 2001

L16 43030 S BURN?
L17 0 S L8 AND L16
L18 553 S L16 AND COMPLEMENT
L19 5 S L16 AND L1
L20 19292 S (BRAIN OR GUT OR INTESTINAL) (3A) ISCHEM?
L21 4 S L20 AND L1
L22 15187 S NEPHRITIS
L23 16065 S NEPHRIT?
L24 150 S L1 AND L23
L25 133 S L22 AND L1
L26 27 S L25 AND PY>1997
L27 36 S L25 AND PY>1996
L28 97 S L25 NOT L27
L29 207 S IMMUNE COMPLEX NEPHRIT?
L30 4 S L1 AND L29

BRS	L1	446	(C adj 1Q) OR (C1 adj Q) OR C1Q OR (COMPLEMENT adj (C1Q OR 1Q))	USPAT	6/4/01 13:59			0
BRS	L2	32	(INHIBIT\$4 OR ANTAGON\$4 OR INACTIVAT\$4) near6 1	USPAT	6/4/01 14:17			0
BRS	L3	151080	infarct\$4 or ((brain or gut or intestinal) near2 ischemi\$4) or stroke\$1 or (rheumatoid near3 arthritis) or lupus or (immune near2 complex near2 nephritis)	USPAT	6/4/01 14:04			0
BRS	L4	16	2 and 3	USPAT	6/4/01 14:16			0
BRS	L5	0	2 and 3	US-PGPUB; EPO; JPO; DERWENT	6/4/01 14:17			0
BRS	L6	32		2 USPAT	6/4/01 14:18			0
BRS	L7	0		2 US-PGPUB; EPO; JPO; DERWENT	6/4/01 14:18			0
BRS	L8	2000	1998gb\$02439.ap,prai.	US-PGPUB; EPO; JPO; DERWENT	6/4/01 14:19		Truncation Overflow. Return string from Server is: 5'0'0'199	1
BRS	L9	0	1998\$gb02439.ap,prai.	US-PGPUB; EPO; JPO; DERWENT	6/4/01 14:20			0
BRS	L10	0	wo9907406.pn.	US-PGPUB; EPO; JPO; DERWENT	6/4/01 14:20			0
BRS	L11	1	schwaeble\$.in. and collectin.ti.	US-PGPUB; EPO; JPO; DERWENT	6/4/01 14:21			0

STIC-ILL

DR180.ES

From: Romeo, David
Sent: Monday, June 04, 2001 12:12 PM
To: STIC-ILL
Subject: 09503421 van den Berg RH, Faber-Krol M, van Es LA, Daha MR.

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE
van den Berg RH, Faber-Krol M, van Es LA, Daha MR.

Regulation of the function of the first component of complement by human C1q receptor.

Eur J Immunol. 1995 Aug;25(8):2206-10.

PMID: 7664783

Rocco H. van den Berg,
Maria Faber-Krol,
Leendert A. van Es and
Mohamed R. Daha

Department of Nephrology,
University Hospital Leiden, Leiden,
The Netherlands

Regulation of the function of the first component of complement by human C1q receptor

A membrane-associated receptor for the C1q subcomponent of complement is widely distributed among different cell types. While a number of possible physiological functions of the C1q receptor (C1qR) on different cell types have been described, the way in which C1qR regulates complement activity remains unclear. This report describes the mechanism by which C1qR regulates activation of the first component of complement, C1. Using purified components of complement, we were able to show that membrane-associated C1qR as well as detergent-solubilized C1qR, purified from polymorphonuclear leukocytes, human umbilical vein endothelial cells or an endothelial cell line, EA.hy 926, are able to inhibit complement-mediated lysis of C1q-sensitized erythrocytes. Using hemolytic assays, we were able to demonstrate that C1qR prevents the association of C1q with C1r and C1s to form macromolecular C1. In addition, incubation of C1qR with the collagen-like stalks, but not with the globular heads of C1q, inhibits the effect of C1qR. This demonstrates that C1qR exerts its complement inhibitory effect by binding to the collagen-like stalk of C1q. No complement regulatory effect of C1qR was observed on preformed macromolecular C1. These data suggest that besides such well-known complement regulatory molecules as CD55 (DAF), CD46 (MCP), CD35 (CR1) and CD59 (HRF), C1qR too is able to regulate complement activity.

1 Introduction

The human complement system plays an important role in the humoral defense against microorganisms and in the initiation of the immune response. Activation of the complement system may occur via the classical or the alternative pathway. Both pathways lead to activation of C3 and to the recruitment and activation of the terminal sequence up to C9, resulting in formation of the membrane attack complex, C5b-C9 [1, 2]. *In vivo* complement activation is regulated by a number of fluid phase inhibitors, such as C1 esterase inhibitor (C1-In) [3], factor H [4] and factor I [5]. In addition, regulation of complement takes place at the tissue level. Membrane molecules such as CD55 (DAF), CD46 (MCP), CD35 (CR1) and CD59 (HRF) have been shown to have important functions in this process [6]. Regulation of C1 activation occurs by C1-In, which is able to react with activated C1r and C1s, present in macromolecular C1 [3, 7, 8]. Besides the regulation of precursor-C1 activity, C1-In is also able to dissociate activated C1r and C1s from activator-bound C1q [9].

Many cell types, such as endothelial cells, platelets, fibroblasts, neutrophils and lymphocytes, express receptors (C1qR) for the collagen-like stalk of C1q [10–19]. C1qR is

able to interact with fluid-phase and activator-bound C1q, such as is found in immune complexes [8, 20, 21]. A functional complement regulatory role for C1qR has not been described. However, since free C1q or activator-bound C1q can potentially react with precursor C1r and C1s to form a new C1 macromolecule [22, 23] we have now assessed how C1qR is able to influence the activity of C1.

In this study we demonstrate that C1qR, isolated from endothelial cells or polymorphonuclear leukocytes, is able to inhibit the formation of C1, thereby regulating activation of the classical pathway of the complement system.

2 Materials and methods

2.1 Purification of C1qR

Endothelial C1qR was isolated from batches of 1×10^6 human umbilical vein endothelial cells (HUVEC, isolated and cultured as described by Ballieux et al. [24]) or the endothelial cell line EA.hy 926 [25]. Cells were washed, disrupted by freeze-thawing five times in buffer A (5 mM sodium phosphate containing 5 mM EDTA, 150 mM NaCl, 10 mM ϵ -amino-n-caproic acid (EACA), 0.5 mM PMSF and 0.5 U Trasylol, pH 7.5) and membranes were obtained by centrifugation for 10 min at $30\,000 \times g$. PMN membranes were isolated as described by Leid et al. [26]. After PMN and endothelial membranes were washed three times, sonicated and incubated for 2 h at 4°C with buffer A, containing 1% (v/v) NP40, the mixture was centrifuged for 20 min at $30\,000 \times g$ to obtain detergent-solubilized C1qR. Supernatant was adjusted to a conductivity of 8 mS by dilution with water, precleared using Sepharose-BSA and applied to a Sepharose-C1q column in buffer A, containing 0.05% (v/v) NP40. After extensive washing, bound material was eluted with a step gradient of 1 M NaCl. In the fractions, C1qR activity (measured as described below), protein content and conductivity were

[I 14261]

Correspondence: Rocco H. van den Berg, Department of Nephrology, Building 1, D3-P, University Hospital Leiden, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands (Fax: 31-71-248118)

Abbreviations: HUVEC: Human umbilical vein endothelial cells EA: Antibody-sensitized erythrocytes C1qD: C1q-depleted serum

Key words: C1q receptor / Complement / Immunoregulation / Endothelial cells / Neutrophils

assessed. Fractions with C1qR activity were pooled, dialyzed against 20 mM Tris-HCl, pH 8.0 and subsequently applied to a RESOURCE Q anion exchange column (Pharmacia-Biotech, Roosendaal, The Netherlands). After washing, elution of bound proteins was carried out using a linear gradient of NaCl and fractions were tested for protein content, conductivity and C1qR activity. Fractions with a conductivity between 15 mS and 20 mS, containing C1qR, were pooled, concentrated using a Speedvac and subjected to gel filtration on Superdex 200 HR 10/30 (Pharmacia). C1qR activity was assessed and positive fractions, coinciding with an apparent molecular mass of 60 kDa, were pooled, aliquoted and frozen at -80°C . By SDS-PAGE analysis one band of 66 kDa was observed after staining with Coomassie blue [27].

2.2 Isolation of C1q

C1q was isolated essentially as described by Wing et al. [28]. In brief, C1q from 2.5 l human serum was precipitated by treatment with PEG-6000 (E. Merck, Amsterdam, The Netherlands) to a final concentration of 3% (w/v) for 1 h at 0°C . After centrifugation, the pellet containing C1q was dissolved in 150 ml Veronal-buffered saline (VBS is 0.142 M NaCl, 5 mM sodium-diethylbarbiturate, pH 7.4), conductivity was adjusted to 12 mS with ice-cold water and EDTA was added to a final concentration of 2 mM. The solution was applied to a rabbit IgG-Sepharose column (prepared by overnight incubation at 4°C of human IgG-Sepharose with an excess of rabbit anti-human-IgG IgG and subsequent washing with PBS). After extensive washing with PBS containing 2 mM EDTA, C1q was eluted with PBS containing 2 mM EDTA and 1 M NaCl. Fractions with C1q content, as determined by a C1q hemolytic assay [29], were pooled, concentrated and fractionated on a Superdex 200 Hiloal 26/60 gel filtration FPLC column (Pharmacia). Fractions were tested for functional C1q activity in a C1q hemolytic assay and positive fractions were pooled, concentrated to 8 mg/ml and stored at 0°C on ice.

2.3 Isolation of C1r and C1s

Human C1r and C1s were purified essentially as described by Peitsch et al. [30], with some modifications. In short, C1 in fresh human serum was precipitated with PEG-6000 with a final concentration of 3% (w/v), centrifuged and the pellet dissolved in VBS containing 5 mM CaCl_2 and 1 mM benzamidine (Aldrich Chemie, Brussels, Belgium). Crude C1 was then applied to a rabbit IgG-Sepharose column and washed with PBS and 1 mM benzamidine. C1r and C1s were dissociated from bound C1q by elution with PBS containing 2 mM EDTA. In the fractions, C1r and C1s were determined by ELISA with antibodies against C1r and C1s (kindly provided by Dr. G. Arlaud, Grenoble, France). While C1r and C1s appeared in one peak, no C1q was found in the fractions as determined by a hemolytic assay. The protein peak containing C1r and C1s was pooled, concentrated and frozen in portions at -80°C . The pool of C1r and C1s contained approximately 50% activated C1r and C1s as determined by trypsin activation [31].

2.4 Isolation of C4

C4 was isolated as described by Kalli et al. [32] with some minor modifications. Fresh human serum was adjusted to 3 mS by addition of ice-cold buffer containing 25 mM sodium phosphate, 100 mM EACA and 25 mM benzamidine, pH 7.5 before it was applied to a Q-Sepharose Fast Flow anion exchange column (Pharmacia). After washing, bound material was eluted with a linear gradient of NaCl. Fractions were tested for both C4 and C1 hemolytic activities. C4-containing fractions, eluting between 13 and 21 mS, were pooled, aliquoted and kept at -80°C until use. No detectable C1 activity was present in the C4 preparation.

2.5 Detection of C1qR

To determine the complement inhibitory capacity of C1qR from endothelial cells or PMN, the following experiment was performed: 1×10^7 sheep erythrocytes (E), sensitized with rabbit IgG anti-E (EA) were incubated with 1/25 diluted C1q-depleted serum (C1qD, [29]), a suboptimal amount of C1q and different dilutions of detergent-solubilized C1qR or equivalent amounts of sonicated PMN membranes in DGVB⁺⁺ (71 mM NaCl, 2.5 mM sodium diethylbarbiturate, 0.05% (v/v) gelatin, 3% (w/v) D-glucose, 1 mM MgCl_2 and 0.15 mM CaCl_2). After 1 h at 37°C , percent lysis was determined relative to a reagent blank and 100% lysis, expressed as units/ml (Z) and converted to percentage of inhibition.

2.6 Influence of C1qR on assembly and activation of C1

To determine the effect of C1qR on EA-bound C1q, EAC1q, (1×10^8 /ml) were incubated with increasing concentrations of C1qR for 30 min at 30°C , washed and subsequently incubated with 1/25 diluted C1qD for 1 h at 37°C . The degree of lysis was determined and expressed as percentage of inhibition.

To examine whether C1qR could react with EA and cause subsequent inhibition of C1q hemolytic activity, EA (1×10^8 /ml) were incubated with increasing concentrations of C1qR for 30 min at 30°C , washed and incubated with a limited dose of C1q in the presence of C1qD. Again after 1 h at 37°C , lysis was assessed and the effect of C1qR expressed as percentage of inhibition.

The effect of C1qR on the assembly of EA-bound C1q with C1r and C1s was assessed as follows: EAC1q, generated by incubation of 1×10^7 EA with 250 $\mu\text{g}/\text{ml}$ C1q for 30 min at 30°C in DGVB⁺⁺, were washed and incubated with various concentrations of C1qR and a fixed dose of C1r and C1s. After washing the intermediates, the amount of C1 assembled on EA was quantified by incubation with 15 ng C4 for 30 min at 30°C . The residual amount of C4 was assessed using a hemolytic assay for C4 [33].

2.7 Interaction of C1qR with collagen-like stalks and globular heads of C1q

Since it has been demonstrated that C1qR interacts with the collagen-like stalk of C1q, EAC1q were incubated with

a fixed amount of solubilized C1qR or equivalent amounts of sonicated PMN membranes and various concentrations of collagen-like stalks or globular heads of C1q (prepared as described by van den Döbelsteen et al. [34]) for 30 min at 30°C. Following addition of a limited amount of C1q in diluted C1qD, samples were incubated for 1 h at 37°C and finally assessed for reversal of C1qR-mediated inhibition of C1q hemolytic activity.

3 Results

The results of the present study demonstrate that sonicated membranes of PMN (with an equivalent amount of 0–20 µg/ml C1qR considering 46 µg C1qR/ 1×10^9 PMN [17]) are able to inhibit C1q hemolytic activity to almost 100% in a dose-dependent fashion (Fig. 1). Preincubating a fixed amount of membranes (equivalent to 1.25 µg/ml C1qR) with increasing concentrations of collagen-like C1q stalks (0–5 µg/ml) before the addition of C1qD and a limited amount of C1q resulted in a dose-dependent reversal of the observed inhibition of C1q hemolytic activity to approximately 75% (Fig. 2). Since co-incubation with globular C1q heads did not reverse the inhibition of C1q activity, this experiment clearly demonstrates that the membrane-associated receptor for the collagen-like C1q stalk (C1qR) is responsible for the observed inhibition of C1q hemolytic activity. In agreement with our previous observations, detergent-solubilized C1qR, derived from either HUVEC or the endothelial cell line EA.hy 926 inhibits C1q activity in a dose-dependent fashion. In addition we found that solubilized C1qR isolated from PMN has a comparable activity to that of C1qR from HUVEC or EA.hy 926 (Fig. 3). All detergent-solubilized C1qR preparations induced a dose-dependent inhibition of C1q hemolytic activity with a maximum of 75–95%.

To obtain more insight into the mechanism of interaction of C1q with C1qR, all of the following experiments were performed with detergent-solubilized C1qR to circumvent effects of other membrane-associated molecules. To determine the effect of the interaction of C1qR with EA-bound C1q, EAC1q were first incubated with different concentrations of C1qR, ranging in dose between 0 and 5 µg/ml, for 30 min at 30°C, washed and subsequently exposed to C1qD for 1 h at 37°C. This experiment revealed a dose-dependent inhibition of C1q activity, suggesting binding of C1qR to EA-bound C1q (Fig. 4).

To exclude a direct effect of C1qR on EA themselves, EA were first incubated with different amounts of C1qR for 30 min at 30°C, washed and incubated with a fixed amount of C1q in C1qD for 1 h at 37°C. A slight binding to EA was observed, causing a subsequent effect on C1q hemolytic activity (Fig. 4).

The effect of C1qR on the assembly of EA-bound C1q with C1r and C1s was assessed by incubating EAC1q with a pool of C1r and C1s in the presence of different concentrations of C1qR. C1qR caused a dose-dependent inhibition of formation of EAC1, as demonstrated by inhibition of C4 consumption (Fig. 5).

To determine whether C1qR also has a direct effect on C1, EAC1 was incubated with different concentrations of

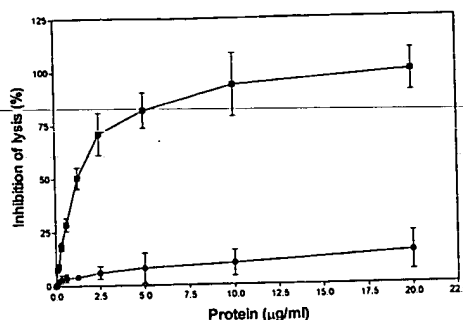


Figure 1. Inhibition of C1q hemolytic activity by sonicated PMN membranes. Different concentrations of sonicated PMN membranes ($0-4.3 \times 10^8$ PMN/ml with an equivalent of 0–20 mg/ml C1qR (—■—) or BSA (—●—) was added to 1×10^7 EA in 1/25 diluted C1qD with a suboptimal amount of C1q. After 1 h incubation at 37°C and centrifugation, A_{414} of the supernatants were measured. Percentage of lysis was determined relative to a reagent blank and 100% lysis and converted to percentage of inhibition as described in Sect. 2.5.

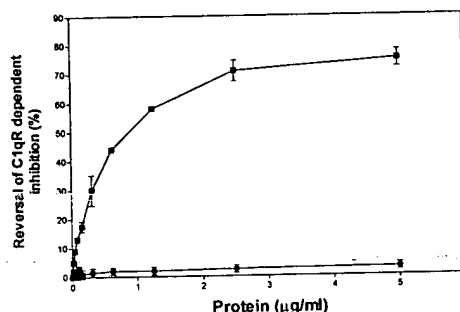


Figure 2. Reversal of PMN membrane inhibitory activity on C1q by co-incubation with collagen-like C1q stalks but not with C1q globular heads. PMN membranes (with an equivalent of 1.25 µg/ml C1qR) were incubated for 30 min at 30°C in the presence of different concentrations of collagen-like C1q stalks (—■—) or globular heads of C1q (—●—). A limited amount of C1q and 1/25 diluted C1qD was added and incubated 1 h at 37°C. Percentage of inhibition of C1q-dependent lysis was determined and presented as percentage of reversal of inhibition relative to that of C1qR preincubated with buffer alone.

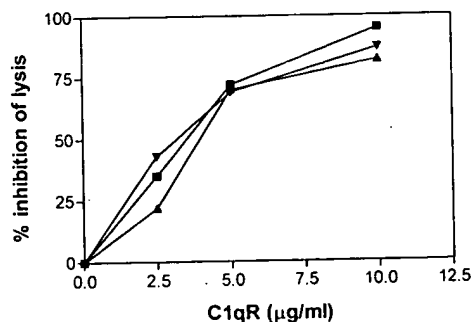


Figure 3. Inhibitory effect of C1qR on lysis of EA in C1qD with suboptimal amounts of C1q. Different dilutions of C1qR, isolated from HUVEC (—■—), the endothelial cell line EA.hy 926 (—▲—) or PMN (—▼—), were incubated with 1×10^7 EA, a limited amount of C1q and 1/25 diluted C1qD. After 1 h incubation at 37°C and centrifugation, A_{414} of the supernatants were measured. Percentage of lysis was determined relative to a reagent blank and 100% lysis and converted to percentage of inhibition as described in Sect. 2.5.

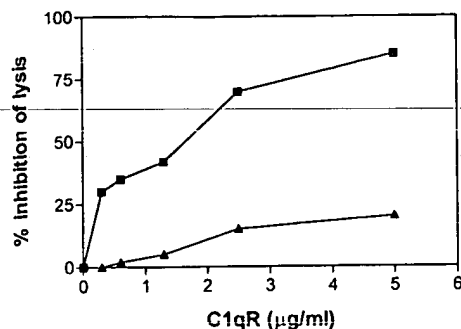


Figure 4. Inhibitory effect of ClqR on lysis of EAC1q in ClqD. EAC1q (1×10^7) were incubated for 30 min at 30°C with different concentrations of ClqR from PMN. After washing 1/25 diluted ClqD was added followed by 1 h of incubation at 37°C. Percentage of inhibition of lysis was determined (■). To determine the direct inhibitory effect of ClqR on EA, EA (1×10^7) were incubated 30 min 30°C with different amounts of ClqR, washed, then incubated with a limited amount of Clq in ClqD for 1 h at 37°C. Inhibition of Clq-mediated lysis was determined as before (▲).

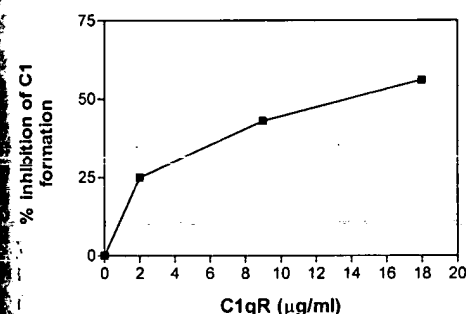


Figure 5. Inhibition of EAC1 formation by ClqR. EAC1q (1×10^7), were incubated for 30 min at 30°C with different amounts of ClqR, isolated from PMN, and a fixed dose of C1r and C1s. After washing, C4 was added and incubated for 30 min at 30°C. Residual C4 was determined as described [33] and the percent inhibition of C1 formation compared to tubes without ClqR was calculated.

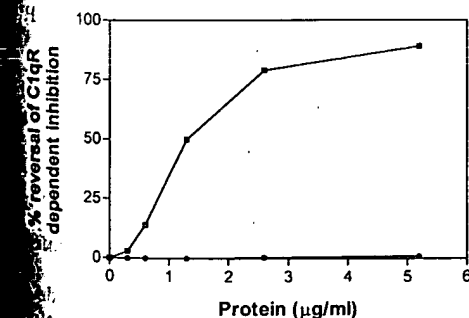


Figure 6. Reversal of ClqR-mediated inhibition of lysis by incubation with collagen-like stalks or globular heads of Clq. EAC1q were incubated with a fixed amount of PMN-derived ClqR in the absence or presence of different amounts of collagen-like stalks (■) or globular heads (○) of Clq for 30 min at 30°C. ClqD diluted 1/25 and a limited amount of Clq were added and incubated for 1 h at 37°C. Percentage of inhibition of Clq-dependent lysis was determined and presented as percentage of inhibition of lysis.

ClqR, washed and the amount of activated C1 assessed by indirect C4 consumption. No detectable effect of ClqR on activated C1 was found (data not shown).

The interaction of ClqR with C1q was analyzed further by incubation of EAC1q with a fixed concentration of ClqR in the presence of different concentrations of collagen-like stalks or globular heads of C1q. Following addition of a limited amount of C1q in ClqD, the degree of reversal of the ClqR-mediated effect was calculated. While collagen-like stalks of C1q caused a dose-dependent reversal of ClqR inhibition, globular heads of C1q had no detectable effect (Fig. 6).

4 Discussion

This study analyzes the mechanism by which ClqR exerts its modulating effect on C1q. The present study indicates that the nearly 100% inhibition of C1q hemolytic activity by PMN membranes is almost exclusively mediated by the membrane-associated receptor for the collagen-like stalks of C1q, ClqR (Figs. 1 and 2). To investigate the mechanism by which ClqR inhibits C1q hemolytic activity, purified detergent-solubilized ClqR was isolated from endothelial cells and PMN. To exclude the possibility that the behavior of detergent-extracted and purified ClqR differs from that of membrane-associated ClqR, detergent-solubilized ClqR from PMN and the cell line EA.hy 926 was tested for its capacity to inhibit C1q hemolytic activity. Fig. 3 shows that all ClqR isolates were able to inhibit C1q hemolytic activity and had a comparable capacity as that which we demonstrated previously for ClqR isolated from endothelial cells [12]. Since detergent-solubilized ClqR from endothelial cells and PMN had comparable activity as membrane-associated ClqR, all subsequent experiments were performed with detergent-solubilized ClqR isolates. Only data from ClqR isolated from PMN are presented since similar results were obtained with endothelial cell derived ClqR.

ClqR exerts its effect on C1q hemolytic activity by binding to C1q (Fig. 4). It is demonstrated that pre-incubation of EAC1q with ClqR followed by washing, inhibits C1q activity in a dose-dependent fashion. The experiments using collagen-like stalks or globular heads of C1q demonstrate that ClqR binds to the collagen-like stalk of C1q. These observations are compatible with the findings of others [11, 16, 35-37] and with our own results [12].

Macromolecular C1 is composed of C1q, C1r and C1s, held together by cations [38]. Preincubation of EAC1q with ClqR and subsequent incubation with a pool of C1r and C1s resulted in a dose-dependent inhibition of formation of C1. We conclude from these experiments that ClqR, by binding to C1q, prevents association of C1q with C1r and C1s. It has been reported that upon activation of C1 in plasma, C1r and C1s are dissociated from C1q by C1-In [9]. This mechanism results in activator-bound C1q. Potentially, this C1q may again interact with precursor C1r and C1s to form a new C1 molecule [39]. ClqR, by preventing this association, may therefore regulate the degree of complement activation on cells bearing ClqR, and thereby suppress amplification of an inflammatory reaction. In addition, it may be that ClqR is shed into the fluid

case, e.g. during inflammation and causes inhibition of complement activation. In this manner, C1qR may be of importance in down-regulation of complement activation in general.

In addition to C1qR, two other human C1q-binding molecules were described, which are both able to inhibit the classical complement route. Decorin, a small collagen-binding dermatan sulfate proteoglycan has a relative molecular mass of 100 kDa and is present as a component of the extracellular matrix on many tissues. Decorin can act as a complement regulatory molecule since it is able to inhibit C4 consumption in the fluid phase by binding to C1q. Like C1qR, it only reacts with isolated C1q and not with C1q in the C1 macromolecule [40]. Since decorin binds to both the collagen-like and globular domains of C1q it may prevent further interactions of C1q with for example C1qR.

More recently a globular C1q receptor of 33 kDa (gC1qR) was isolated from Raji cells [41]. gC1qR binds to the globular heads of C1q and was reported to inhibit complement activation in the fluid phase. The complement inhibitory mechanism is not known yet, but it is possible that gC1qR interferes with the interaction of C1q with IgG.

Besides C1q-binding molecules, many other complement regulators exist that are either membrane bound or found in the circulation (for review see [6]). Compared with C1qR, which acts at the earliest phase of complement activation, most complement regulators interfere with complement activation in a later phase in the complement activation sequence. For this reason, C1qR may be of importance at the onset of an inflammatory response.

The authors thank Evert Heemskerk for the isolation of C1q and culture of HUVEC. This study was sponsored by the Netherlands Organization for Scientific Research.

Received March 15, 1995; in revised form May 15, 1995; accepted June 7, 1995.

5 References

- Podack, E. R., Kolb, W. P. and Müller-Eberhard, H. J., *J. Immunol.* 1976. 116: 1431.
- Kolb, W. P. and Müller-Eberhard, H. J., *J. Exp. Med.* 1975. 141: 724.
- Ratnoff, O. D., Pensky, J., Ogston, D. and Naff, G. B., *J. Exp. Med.* 1969. 129: 315.
- Zipfel, P. F. and Skerka, C., *Immunol. Today* 1994. 15: 121.
- Pangburn, M. K. and Müller-Eberhard, H. J., *Proc. Natl. Acad. Sci. USA* 1978. 75: 2416.
- Morgan, B. P. and Meri, S., *Springer Semin. Immunopathol.* 1994. 15: 369.
- Hughes-Jones, N. C. and Gorick, B. D., *Mol. Immunol.* 1982. 19: 1105.
- Cooper, N. R., *Adv. Immunol.* 1985. 37: 151.
- Ziccardi, R. J. and Cooper, N. R., *J. Immunol.* 1979. 123: 788.
- Ghebrehiwet, B., *Behring Inst. Mitt.* 1989. 84: 204.
- Peerschke, E. I. B. and Ghebrehiwet, B., *J. Immunol.* 1990. 145: 2984.
- Daha, M. R., Dunn, L., van den Berg, R., Muizert-de Lange, Y., Gerritsen, A. and van Es, L. A., *Med. Inflamm.* 1993. 2: 447.
- Crockard, A. D., Thompson, J. M., Malhotra, R. and McNeill, T. A., *Immunol. Lett.* 1993. 36: 195.
- Andrews, B. S., Shadforth, M., Cunningham, P. and Davis, J. S., IV, *J. Immunol.* 1981. 127: 1075.
- Ghebrehiwet, B., Silvestri, L. and McDevitt, C., *J. Exp. Med.* 1984. 160: 1375.
- Bordin, S., Kolb, W. P. and Page, R. C., *J. Immunol.* 1983. 130: 1871.
- Bobak, D. A., Frank, M. M. and Tenner, A. J., *J. Immunol.* 1986. 136: 4604.
- Chen, A., Gaddipati, S., Hong, Y., Volkman, D. J., Peerschke, E. I. B. and Ghebrehiwet, B., *J. Immunol.* 1994. 153: 1430.
- Lin, T.-Y. and Fletcher, D. S., *J. Biol. Chem.* 1980. 255: 7756.
- Daha, M. R., Miltenburg, A. M. M., Hiemstra, P. S., Klar-Mohamad, N., van Es, L. A. and van Hinsbergh, V. W. M., *Eur. J. Immunol.* 1988. 18: 783.
- Ziccardi, R. J. and Cooper, N. R., *J. Immunol.* 1977. 118: 2047.
- Siegel, R. C., Schumaker, V. N. and Poon, P. H., *J. Immunol.* 1981. 127: 2447.
- Ballieux, B. E. P. B., Zondervan, K. T., Kievit, P., Hagen, E. C., van Es, L. A., van der Woude, F. J. and Daha, M. R., *Clin. Exp. Immunol.* 1994. 97: 52.
- Edgell, C.-J. S., McDonald, C. C. and Graham, J. B., *Proc. Natl. Acad. Sci. USA* 1983. 80: 3734.
- Leid, R. W., van der Heijden, I. and Ballieux, B. E. P. B., *Eur. J. Immunol.* 1993. 23: 2939.
- Laemmli, U. K., *Nature* 1970. 227: 680.
- Wing, M. G., Seilly, D. J., Bridgman, D. J. and Harrison, R. A., *Mol. Immunol.* 1993. 30: 433.
- Kolb, W. P., Kolb, L. M. and Podack, E. R., *J. Immunol.* 1979. 122: 2103.
- Peitsch, M. C., Kovacsics, T. J. and Isliker, H., *J. Immunol. Methods* 1988. 108: 265.
- Valet, G. and Cooper, N. R., *J. Immunol.* 1974. 112: 339.
- Kalli, K. R. and Fearon, D. T., *J. Immunol.* 1994. 152: 2899.
- Gaither, T. A., Alling, D. W. and Frank, M. M., *J. Immunol.* 1974. 113: 574.
- van den Dobbelen, M. E. A., van der Woude, F. J., Schreijers, W. E. M., Klar-Mohamad, N., van Es, L. A. and Daha, M. R., *J. Immunol.* 1993. 151: 4315.
- Wautier, J. L., Souchon, H., Reid, K. B. M., Peltier, A. P. and Caen, J. P., *Immunochemistry* 1977. 14: 763.
- Guan, E., Robinson, S. L., Goodman, E. B. and Tenner, A. J., *J. Immunol.* 1994. 152: 4005.
- Bordin, S., Smith, M., Ghebrehiwet, B., Oda, D. and Page, R. C., *Clin. Immunol. Immunopathol.* 1992. 63: 51.
- Ziccardi, R. J., *J. Biol. Chem.* 1983. 258: 6187.
- Doekes, G., van Es, L. A. and Daha, M. R., *J. Immunol.* 1983. 131: 1924.
- Krumdieck, R., Hook, M., Rosenberg, L. C. and Volanakis, J. E., *J. Immunol.* 1992. 149: 3695.
- Ghebrehiwet, B., Lim, B.-L., Peerschke, E. I. B., Willis, A. C. and Reid, K. B. M., *J. Exp. Med.* 1994. 179: 1809.

STIC-ILL

mic
PR180.15
Adonab

From: Romeo, David
Sent: Monday, June 04, 2001 11:59 AM
To: STIC-ILL
Subject: 09503421 Malhotra R, Willis AC, Jensenius JC, Jackson J, Sim RB.

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE
Malhotra R, Willis AC, Jensenius JC, Jackson J, Sim RB.

Structure and homology of human Clq receptor (collectin receptor).

Immunology. 1993 Mar;78(3):341-8.

PMID: 8478019

Structure and homology of human C1q receptor (collectin receptor)

R. MALHOTRA, A. C. WILLIS, J.-C. JENSENIUS,* J. JACKSON† & R. B. SIM

Medical Research Council Immunochimistry Unit, Department of Biochemistry, University of Oxford, U.K.,

*Institute of Medical Microbiology, Bartholins Building, University of Aarhus, Aarhus, Denmark and

†Department of Immunology, St James Hospital, Dublin, Ireland

Accepted for publication 1 November 1992

SUMMARY

In this paper we report partial amino acid sequence for C1q receptor (C1qR). The N-terminal amino acid sequence of isolated C1qR and the sequences of peptides obtained by V8/trypsin digestion show a high degree of similarity to the cDNA-derived amino acid sequence of a human protein which was initially reported as a component of RoSSA and subsequently as calreticulin. This sequence in turn shows homology with *Onchocerca volvulus* antigen (RAL-1) and B50 murine melanoma antigen. A component of approximately 53,000 MW, isolated from human spleen, was found to have identical mobility on SDS-PAGE to C1qR and identical N-terminal sequence, but a different overall charge. Human antibodies from Sjögren's syndrome patients did not recognize C1qR, but showed positive reaction with the purified 53,000 MW component from spleen. Rabbit antibodies against denatured C1qR, in contrast, recognized both C1qR and the purified 53,000 MW component. The 53,000 MW spleen component thus has an identical N-terminal sequence to calreticulin, and to the reported RoSSA component, and is recognized by antibodies in Sjögren's syndrome sera. The data obtained indicate that C1qR and the reported calreticulin/RoSSA component are similar but not identical molecules, which belong to the same protein superfamily.

INTRODUCTION

The existence of a cell surface receptor for the C1q complement component was first suggested by Dickler and Kunkel.¹ C1q receptor (C1qR) activity has been reported on most leucocytes, endothelial cells, fibroblasts and platelets² and binding of C1q to its receptor has been reported to mediate a range of phenomena, including phagocytosis, modulation of cytokine and immunoglobulin secretion, and polymorphonuclear leucocyte-endothelium interaction.³ Erdei and Reid⁴ extracted radioactive C1qR from surface-radioiodinated U937 cells and in biosynthetic labelling studies using [³⁵S]cysteine and [³⁵S]methionine they showed that C1qR is synthesized by U937 cells. The molecular species identified as C1qR by Erdei and Reid⁴ has been purified from human tonsil lymphocytes, U937 cells and human spleen.^{5,6} C1qR purified from these sources is a protein of molecular weight 56,000, as assessed by SDS-PAGE under reducing conditions. C1qR is an acidic glycoprotein with 15-20% carbohydrate⁶ and the detergent-solubilized protein behaves as an elongated dimer of molecular weight

115,000 ± 7000. Purified C1qR was found to inhibit the binding of radioiodinated C1q to U937 cells.⁷ Three other proteins, conglutinin, lung surfactant protein A and mannan-binding protein, were found to interact with purified C1qR.⁷ These three proteins, like C1q, have been reported to be involved in phagocytosis and have similar structural organization to that of C1q.⁸ The interaction of these four ligands with C1qR indicates that their opsonic activity may arise through interaction of these proteins with C1qR. Due to the structural and functional similarities between conglutinin, lung surfactant protein A, C1q and mannan-binding protein and the complement association or carbohydrate-binding characteristics of these ligands, we earlier proposed the name collectins for the ligands of C1qR,⁹ and the term collectin receptor for C1qR. Although the binding characteristics and cellular distribution of C1qR are documented and possible physiological roles of C1qR have been reported by a number of workers, no primary structure data have been reported in the past. In this paper we present peptide sequence for the collectin receptor and report on the sequence homology of C1qR with other proteins.

MATERIALS AND METHODS

Purification of C1qR

C1qR was purified from tonsil lymphocytes by the method of Malhotra and Sim,⁶ except that the sample was further processed on a TSKgel DEAE-NPR (4.6 × 35 mm) high-perfor-

Abbreviations: BSA, bovine serum albumin; C1qR, C1q receptor; PBS, phosphate-buffered saline; 8.2 mM Na₂HPO₄/1.5 mM KH₂PO₄ buffer; pH 7.5, containing 139 mM NaCl and 3 mM KCl.

Correspondence: Dr R. Malhotra, Medical Research Council Immunochimistry Unit, Dept. of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

mance liquid chromatography (HPLC) column (TOSHAAS, Philadelphia, PA), pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The bound protein was eluted with a 20-ml linear gradient (flow rate, 1 ml/min) of 0–500 mM NaCl in Tris-HCl buffer (pH 8.0). This step removed detergent as well as removing minor contaminants. Purification of C1qR was monitored by a functional assay (ligand-binding) as described previously.^{6,7} C1qR was radioiodinated using a standard Iodogen reaction.¹⁰

Purification of RoSSA

Human spleen (50 g) was homogenized in 50 ml of 0.01 M sodium phosphate buffer (pH 7.2) containing 150 mM NaCl. The homogenate was centrifuged at 40,000 *g* for 2 hr and RoSSA antigen was purified from the supernatant by the method of Wu *et al.*¹¹ The material was further purified using high-pressure gel ion-exchange filtration, exactly as described for C1qR above, to isolate a homogeneous component of approximately 53,000 MW, termed by us 'RoSSA'.

To determine whether C1qR could be purified from the same source, the pellet from the homogenized spleen was subjected to detergent extraction. The pellet was redissolved in 10 mM phosphate buffer (pH 7.4), containing 1% (w/v) Nonidet P-40, 100 µg of soya bean trypsin inhibitor/ml, 5 mM iodoacetamide, 2.5 mM di-isopropyl phosphorofluoridate, 20 µM 1,10-phenanthroline and 5 µg of pepstatin/ml. The detergent-soluble material was loaded on to a 1-ml Mono Q fast protein liquid chromatography (FPLC) column (Pharmacia), pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.4), containing 0.1% (w/v) emulphogene BC720 (Sigma, Poole, U.K.). Bound proteins were eluted with a linear gradient of 0–1 M NaCl as described previously.⁶ The C1qR pool from Mono Q was then separated from minor contaminants on a TSKgel DEAE-NPR HPLC column, as described earlier.

Large-scale partial purification of crude RoSSA

Larger quantities of human RoSSA were purified from approximately 200 g of human spleen following the method of Venables *et al.*¹² Fractions containing RoSSA antigen were identified by counter-current immunoelectrophoresis. This material was used to make RoSSA-Sepharose resin for adsorption of antibody preparations. Subsequent analysis of the material on a TSKgel DEAE-NPR HPLC column, as above, showed that it did not contain component 2 identified in Fig. 3.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli.¹³ Samples were prepared (reduced or/and alkylated) as described by Fairbanks *et al.*¹⁴ Proteins were detected with Coomassie Brilliant Blue.¹⁴

Immunization of rabbits

Rabbits were injected in the popliteal node region with C1qR (20 µg) in polyacrylamide slices excised from SDS-PAGE. The acrylamide was homogenized and mixed with 1 ml of complete Freund's adjuvant (CFA). Three booster injections each of 60 µg of soluble purified C1qR in 1 ml of incomplete Freund's adjuvant (IFA) were given over a period of 3 months.

Purification of antibodies

Anti-C1qR immunoglobulins were purified from pooled rabbit antiserum by triple sodium sulphate precipitation.¹⁵ Purified antibody was dialysed against phosphate-buffered saline (PBS).

Sjögren's syndrome sera

Human sera positive for anti-RoSSA were obtained from patients with primary Sjögren's syndrome. Sera were analysed for antibody against RoSSA and LaSSB at the Danish State Serum Institute by the standard technique involving Ouchterlony immunodiffusion against extract of human spleen (RoSSA) or extract of rat thymus (LaSSB), and involving suitable positive control standard sera. The sera selected for use in the present study showed positive reaction with RoSSA.

Immunoblotting (Western blot analysis)

Proteins to be immunoblotted were run on SDS-PAGE under non-reducing conditions, and transferred to nitrocellulose (Hybond C, Amersham International, Amersham, U.K.). Antigens were detected using first antibodies and second antibody-alkaline-phosphatase conjugates. The blot was developed as described by Kölbl *et al.*¹⁶

Immunoassay (antigen capture assays)

Microtitre plate wells were coated with 100 µl of purified rabbit anti-C1qR antibody (50 µg/ml) in PBS and non-specific binding sites were blocked with bovine serum albumin (BSA) (3 mg/ml) in PBS. Different dilutions of C1qR (50 µl: max conc. 50 µg/ml) in PBS were mixed with 50 µl of ¹²⁵I-labelled C1qR (2.5 µg/ml; total c.p.m. loaded/well 1.2 × 10⁶) and added to the antibody-coated wells. BSA (50 µl: 50 µg/ml) along with radiolabelled C1qR were loaded on the plate as a control. The plate was incubated for 2 hr at ambient temperature (18–22 °C). The plate was washed extensively with PBS. Bound radioactivity was solubilized and removed with 300 µl of 4 M NaOH.

Agglutination of U937 cells

U937 cells were grown in 50-ml culture flasks under standard conditions.¹⁷ Cells (~10⁷) were washed three times with PBS and were suspended in 1 ml of PBS. The cell pellet obtained from 200 µl of this cell suspension was resuspended in 200 µl of purified anti-C1qR immunoglobulin (0.7 mg/ml) in PBS containing 0.1% (w/v) sodium azide and incubated for 30 min at ambient temperature. Immunoglobulin (0.7 mg/ml) purified from the serum of the rabbit prior to immunization was used as a negative control. Cells were washed three times with 1 ml of PBS containing 0.1% (w/v) NaN₃. To the cell pellet was added 100 µl of goat anti-rabbit IgG (500 µg/ml) in PBS containing 0.1% (w/v) NaN₃ and the cell suspension was incubated for 30 min at ambient temperature. After extensive washing in PBS the cells were suspended in 1 ml of PBS. The cell suspension was observed for agglutination by light microscopy.

Fluorescence detection of bound antibodies

U937 cells (~10⁷) were washed, resuspended and treated with anti-C1qR immunoglobulin as described earlier. Immunoglobulins (0.7 mg/ml) purified from the serum of the rabbit prior to immunization or purified rabbit antibody raised against complement component C3 was used as a negative control. Cells were washed three times with 1 ml of PBS/NaN₃. After washing three times with PBS the cells were incubated with anti-rabbit

immunoglobulins conjugated with fluorescein isothiocyanate (FITC) (100 μ l; diluted in PBS/NaN₃ according to the value recommended by the manufacturer: Serotec, Kidlington, U.K.). FITC-labelled cells were observed and photographed by fluorescence microscopy using a Zeiss Axioskop microscope.

Coupling of partially purified RoSSA to CNBr-activated Sepharose 4B and fractionation of anti-ClqR antibodies

Coupling of partially purified RoSSA to CNBr-activated Sepharose 4B (Pharmacia, Milton Keynes, U.K.) was carried out in 50 mM potassium phosphate buffer (pH 8.2) by the method described by the manufacturer. Protein, 1.5 mg, was coupled per ml of Sepharose. The resin was washed twice with five volumes of coupling buffer followed by a wash with 50 ml of 1 M NaCl. Unreacted binding sites were blocked with 0.1 M ethanolamine-HCl buffer (pH 8.5). RoSSA-Sepharose 4B (3.5 ml) was pre-equilibrated in PBS and poured into a column. Rabbit anti-ClqR antiserum (1 ml) was loaded on to the affinity column, and the column was washed with PBS, until the OD₂₈₀ of the eluate was reduced to background. The column was washed with 6 ml of deionized water and the bound IgG was eluted with 3 M MgCl₂. The material which did not bind to the RoSSA-Sepharose was pooled and designated as 'ClqR-specific' antiserum, whereas the fractions eluted with 3 M MgCl₂ were designated as 'RoSSA cross-reactive' antibodies.

Reduction and alkylation of proteins

Samples for sequence analysis were denatured by the addition of 7.5 M guanidium chloride, 0.125 M Tris-HCl (pH 8.0) and reduced by the addition of dithiothreitol at a final concentration of 6.5 mM. The samples were incubated for 1 hr at 37°C, then 4-vinylpyridine was added to a final concentration of 88 mM to block free cysteine residues. The samples were further incubated for 1 hr at 37°C, dialysed exhaustively against 10 mM NH₄OH, and freeze dried.

Amino acid sequencing

Purified ClqR was reduced and alkylated and the N-terminal amino acid sequence was obtained using an Applied Biosystems 470A protein sequencer and Applied Biosystems 120A analyser. ClqR was found to be highly resistant to trypsin digestion. Therefore, the protein was digested with *Staphylococcus aureus* V8 protease (BCL, Lewes, U.K.). Purified ClqR (200 μ g) was reduced and alkylated and dialysed against 0.1% (w/v) ammonium bicarbonate. The protein was incubated with 5% (w/w) V8 protease for 2 h at 37°C. The peptides were separated on a C₁₈ reverse-phase HPLC column, pre-equilibrated in 0.1% trifluoroacetic acid (TFA). Peptides were eluted with an acetonitrile gradient (0–100%) in 0.1% TFA. The amino acid composition of purified peptides was analysed by HPLC with the Waters Pico-Tag system (Millipore, Watford, U.K.) after hydrolysis in 6 M HCl (6 hr at 125°C). Appropriate peptides were subjected to sequence analysis as above.

In a second set of experiments purified ClqR (50 μ g) was reduced and alkylated and dialysed against 0.1% (w/v) ammonium bicarbonate. The protein was incubated with 2 μ g of V8 protease for 16 hr at 37°C, followed by 16 hr incubation at 37°C with 2 μ g of TPCK-treated trypsin. The peptides generated were purified on a Vydac C₁₈ HPLC column (250 \times 4.6 mm), pre-equilibrated in 0.1% TFA. Peptides were eluted with a linear gradient of 4–72% acetonitrile in 0.1% TFA.

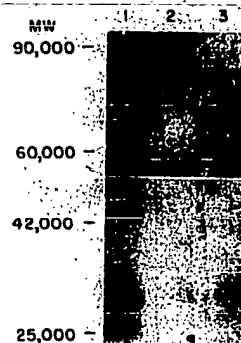


Figure 1. Analysis by SDS-PAGE of different 53,000 MW components isolated from spleen and tonsils. These were run on a 10% gel, and are non-reduced. The major components are all of apparent molecular weight 53,000. Lane 1, RoSSA purified by the method of Wu *et al.*¹¹ without the further purification described in Materials and Methods; lane 2, component 2 from the RoSSA preparation, found to co-run with ClqR on the TSKgel DEAE-NPR HPLC column; lane 3, ClqR purified from tonsils.

RESULTS

N-terminal sequence of ClqR

ClqR was purified as described in Materials and Methods. Purified material, as analysed on SDS-PAGE, under non-reducing conditions, is shown in Fig. 1 (lane 3). A sequence of 14 amino acids at the N-terminal of ClqR was obtained (Table 1). This sequence was found to be identical to the N-terminal sequence of a protein reported to be a component of the autoantigen RoSSA¹⁸ (Table 1).

Peptide sequence

To obtain peptides for sequencing, purified ClqR was digested with *Staphylococcus aureus* V8 protease or with V8 protease and trypsin. Nine of the total of 12 peptides sequenced had sequences identical to segments of the reported RoSSA component^{18,19} (Table 1). Two of the remaining three peptides show similarity to the RoSSA component¹⁹ and the other peptide sequence is not present in the reported sequence of the RoSSA component¹⁹ (Table 1). As noted in Table 1, there is clear quantitative evidence that the peptides which do not match the RoSSA sequence are not derived from contaminants.

Specificity of anti-ClqR antibodies

The specificity of antisera raised against ClqR was established by radioimmunoassay and by Western blot analysis. Radioimmunoassay was performed as described in Materials and Methods. Different dilutions of unlabelled ClqR and BSA were used as potential inhibitors for binding of radioiodinated ClqR to anti-ClqR antibodies. Concentration-dependent inhibition of binding of ¹²⁵I-labelled ClqR to microtitre plate-bound anti-

Table 1. Amino acid sequences of C1qR peptides. Except for the sequences obtained for peptides 1, 2 and 3, all the other peptide sequences are identical to segments of the reported RoSSA component.¹⁹ On a quantitative basis peptides 1 and 2 (yield at first sequence step 70 pm each; not present in RoSSA) were isolated in the same quantity as peptide 6 (initial yield 79 nm; present in the RoSSA sequence); all the three peptides were generated by V8 digest and were from the same batch of digest. This is a strong indication that peptides 1 and 2 are not derived from contaminants

Peptide no.	Peptide sequence	Sequence identity or similarity with RoSSA (region)
N-terminal of C1qR	EPAVYFKEQFLDGD	position 1-14
Peptides generated by V8 protease digest		
1	YKGRQT	Two KG-QT sequences in RoSSA
2	DNQSENMS	not present in RoSSA
3	KPADMS-S	KPEDWDPE sequence present in RoSSA
4	QFLDGDG-TS	position 9-18
5	IDNPE	position 278-282
6	DNPEYSPDPSIYAYDNFDVL-L	position 279-300
7	PDPSIYAYDNFDVL	position 285-298
Peptides generated by Trypsin/V8 protease digest		
8	KPEDWDEEMDGEWEPPIQN	position 233-253
9	GLDLWQVK	position 299-306
10	IDNPEYSPDPSIY	position 278-290
11	VKIDNSQVE	position 168-176
12	FTVK	position 79-82

C1qR was observed in the presence of unlabelled purified C1qR (Fig. 2a), whereas BSA did not affect the binding of radiolabelled C1qR to the antibody. This confirms the recognition of ¹²⁵I-labelled C1qR and unlabelled C1qR by the antibody preparation. Partially purified C1qR from detergent-soluble material from the spleen (after Mono Q FPLC column chromatography) was separated by SDS-PAGE (Fig. 2b; lane 1) and blotted on nitrocellulose filters as described in Materials and Methods. The blots were probed with anti-C1qR antiserum and rabbit anti-C3 antiserum as well as with immunoglobulin from the control preimmunization bleed of an anti-C1qR rabbit. A single band corresponding to C1qR was recognized by the anti-C1qR serum (Fig. 2b, lane 2), but no band with similar MW was recognized by the control antisera (not shown).

Purification of RoSSA

C1qR was found to have a high degree of primary sequence identity with a protein reported initially to be one of the components of the complex RoSSA autoantigen¹⁹ (Table 1). Subsequent work²⁰ has indicated that the cDNA sequence presented by McCauliffe *et al.*¹⁹ may correspond to human calreticulin, and there is further uncertainty as to whether the cDNA sequence does represent a component of RoSSA.²⁰ The function and the cellular localization of the proteins (RoSSA component or calreticulin) encoded by the cDNA sequence^{19,20} are uncertain. C1qR, in contrast, has been isolated on the basis of a highly defined function^{5,6} and is expressed on the cell surface. However, C1qR is clearly closely related to the cDNA-defined sequence.^{19,20} In order to investigate further the relationship between C1qR and the related species, RoSSA was partially purified from a spleen extract by a standard method.¹¹ Wu *et al.*¹¹ reported isolation of two polypeptides with MW of

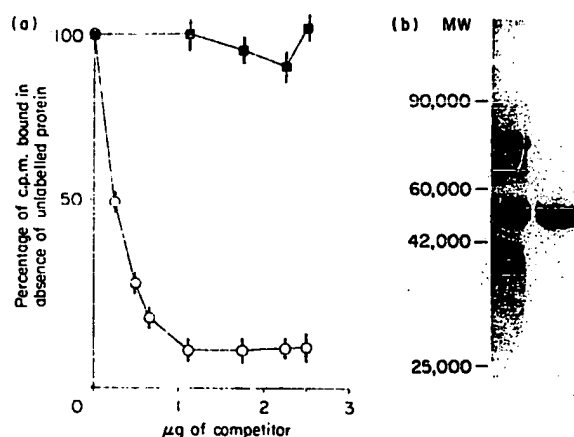


Figure 2. Specificity of anti-C1qR antibodies. (a) Binding of radiolabelled C1qR to solid phase immobilized anti-C1qR antibodies in the presence of unlabelled C1qR or BSA. Different dilutions of unlabelled C1qR (O) were mixed with radiolabelled C1qR in PBS and loaded on to microtitre plate wells coated with purified anti-C1qR antibody. BSA (□) mixed with radiolabelled C1qR was loaded as a control. After extensive washing the bound c.p.m. were measured, as described in Materials and Methods. Control antibodies, namely purified rabbit antibodies against C3 and immunoglobulins purified from serum of the rabbit prior to immunization with C1qR, did not recognize radioiodinated C1qR (not shown). Results of a single experiment with the average and range of triplicate experimental points are shown. (b) Western blot analysis of partially purified C1qR with anti-C1qR antibody. Partially purified C1qR from detergent solubilized extract from spleen was separated by SDS PAGE and blotted on nitrocellulose filters. Lane 1, Coomassie Blue-stained gel track; lane 2, blot probed with purified anti-C1qR antibody. A single band corresponding to C1qR was detected.

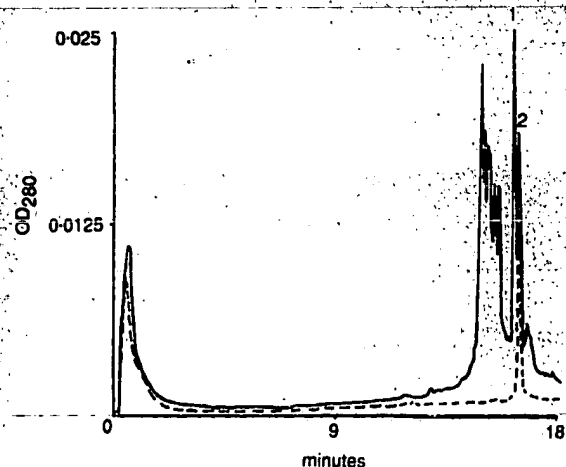


Figure 3. Elution profile of purified C1qR (---) from tonsils and partially purified RoSSA (—) from the TSKgel DEAE-NPR HPLC column. Only peaks 1 and 2 contained material of apparent molecular weight 53,000 (unreduced).

approximately 60,000 and 55,000, as analysed on SDS-PAGE under reducing conditions. Both these molecules were reported to react with human anti-RoSSA antibodies. Using the same method, an apparent single major polypeptide of MW 53,000 (Fig. 1, lane 1) under non-reducing conditions was obtained by us, which showed positive reaction in Western blotting with anti-RoSSA serum. C1qR also migrates as a 53,000 MW protein on SDS-PAGE under non-reducing conditions. The fractions containing RoSSA antigen were further purified on the TSKgel DEAE-NPR HPLC column as described in Materials and Methods. The 53,000 MW material was found to separate into two components of apparently identical MW, component 1 (major) and 2 (minor) (Fig. 3), on the HPLC column. The minor component, component 2 (Fig. 1, lane 2), was found to co-elute with C1qR activity (Fig. 3), while the other was less acidic. The N-terminal sequence (up to 12 residues) of both the components was found to be identical to the N-terminal sequence of C1qR purified from tonsils (Table 1), which in turn is identical to the reported N-terminal sequences of a RoSSA component^{18,19} and calreticulin.²⁰ Of these two components, only component 1 was recognized by human antibodies in Sjögren's syndrome sera. We therefore interpret component 1 as being an appropriate candidate for a component of RoSSA autoantigen, while component 2 is C1qR, as determined by functional assays.^{5,9} Component 1 is referred to hereafter as 'RoSSA'.

Purification of C1qR from detergent solubilized spleen lysate

The pellet obtained from the spleen homogenate after the standard RoSSA extraction method was redissolved in detergent-containing buffer and the procedure for purification of C1qR was followed. Analysis of partially purified C1qR on SDS-PAGE under non-reducing conditions is shown in Fig. 2b (lane 1). C1qR from spleen migrated identically to tonsil C1qR on the TSKgel DEAE-NPR HPLC column (Fig. 3).

Western blot analysis and antibody binding of 'RoSSA' and C1qR

The results above indicate that C1qR has considerable but not

complete sequence identity to the RoSSA component reported by McCauliffe *et al.*¹⁹ and has the same N-terminal sequence as an anti-RoSSA reactive protein (Fig. 3, component 1) isolated by us from spleen. However, C1qR does not co-elute with 'RoSSA' on the TSK-DEAE HPLC column, and can be differentially extracted from spleen. To investigate the similarities between C1qR and 'RoSSA', Western blot analysis of the different molecules of apparent MW 53,000 (non-reducing) isolated from spleen and tonsils was performed. The proteins were blotted onto nitrocellulose. Different sets of filters were treated with Sjögren's syndrome sera from any of eight patients or with anti-C1qR antiserum and the blots were developed with alkaline phosphatase conjugated anti-human IgG and anti-rabbit IgG respectively. The results of the experiments are shown in Table 2. All the 53,000 MW proteins showed positive reaction with anti-C1qR antibody, whereas antibodies from Sjögren's syndrome sera only interacted with purified 'RoSSA' (component 1). To investigate further the antigenic differences between C1qR and 'RoSSA', anti-C1qR antiserum was fractionated on a RoSSA-Sepharose 4B affinity column made with partially purified RoSSA. Purified C1qR and 'RoSSA' were separated on SDS-PAGE and were blotted on nitrocellulose filters. Different sets of filters were incubated with unfractionated anti-C1qR, and with the putative C1qR-specific and RoSSA cross-reactive fractions obtained from RoSSA-Sepharose. Unfractionated anti-C1qR, C1qR-specific and RoSSA cross-reactive material showed positive reaction with C1qR (Table 2), whereas C1qR-specific showed no reaction with 'RoSSA' (Table 2). Therefore the anti-C1qR antiserum can successfully be absorbed to remove specificity for 'RoSSA' without complete removal of anti-C1qR activity.

To establish further the differences between C1qR and 'RoSSA', the binding of purified C1qR and 'RoSSA' to solid phase immobilized anti-RoSSA immunoglobulins (i.e. the immunoglobulin fraction from Sjögren's syndrome sera), anti-C1qR immunoglobulins and control immunoglobulins was investigated. Microtitre plate wells were coated with 100 µl of purified rabbit anti-C1qR antibodies (50 µg/ml) or purified human anti-RoSSA antibodies (50 µg/ml) or purified rabbit anti-ovalbumin antibodies (50 µg/ml) in PBS. Non-specific binding sites were blocked with BSA (3 mg/ml) in PBS. ¹²⁵I-labelled C1qR or ¹²⁵I-labelled 'RoSSA' in PBS was loaded on to the immunoglobulin-coated wells and incubated for 24 hr at 4 °C. After extensive washings with PBS the bound c.p.m. were measured as described in Materials and Methods. The results of the binding experiments are shown in Fig. 4. Radioiodinated 'RoSSA' was found to bind both to anti-C1qR and to anti-RoSSA, whereas radioiodinated C1qR did not bind significantly to anti-RoSSA. The Western blot analysis and the solid phase binding studies indicate that C1qR and 'RoSSA' have antigenic differences.

Agglutination of U937 cells by anti-C1qR antibodies

McCauliffe *et al.*¹⁹ stated, without presenting experimental data, that the antibodies raised against the amino terminal portion of the protein encoded by their cDNA sequence (a sequence segment identical to the N-terminus of C1qR), locate only an intracellular species in mouse L cells (fibroblasts) and human Hep-2 cells (epithelioid cells). Erdei and Reid⁴ however, showed, by surface radioiodination of U937 cells, that C1qR is located

Table 2. Cross-reactivity between RoSSA and C1qR. Western blot analysis of different 53,000 MW components isolated from spleen and tonsils with anti-C1qR antiserum and anti-RoSSA antiserum. Materials blotted were components 1 and 2 from the spleen RoSSA preparation, isolated C1qR from tonsils (co-running with component 2 on HPLC) and the C1qR extract from spleen after the FPLC mono Q step of purification

Antiserum	Blotted protein			
	RoSSA component 1 from spleen	C1qR (tonsils)	Partially purified C1qR (detergent solubilized spleen extract)	Component 2 from the spleen RoSSA preparation
Anti-RoSSA antiserum (human)	+++	---	---	---
Antiserum to denatured C1qR (rabbit)	+++	+++	+++	+++
Antiserum to denatured C1qR (rabbit), not bound to RoSSA-Sepharose	---	+++	ND*	ND
Antiserum to denatured C1qR (rabbit), which bound to RoSSA-Sepharose	+++	+++	ND	ND

* ND, not done.

on the cell surface. Purified soluble C1qR also inhibits the binding of radiolabelled C1qR to U937 cells, providing further evidence for the surface localization of C1qR. To examine further the cellular localization of C1qR, U937 cells were treated with purified anti-C1qR antibodies as described in Materials and Methods. In the presence of anti-C1qR strong aggregation of U937 cells was observed (Fig. 5a), whereas in the presence of non-immune rabbit immunoglobulins, the cells were not agglutinated (Fig. 5b). In a second set of experiments U937 cells were treated with anti-C1qR antibody or non-immune rabbit immunoglobulins, followed by interaction with FITC-labelled anti-rabbit IgG. Figure 5c shows that in the intact cells specific

plasma membrane immunofluorescence was observed. This confirms that the anti-C1qR antibodies are recognizing an antigen exposed on the cell surface.

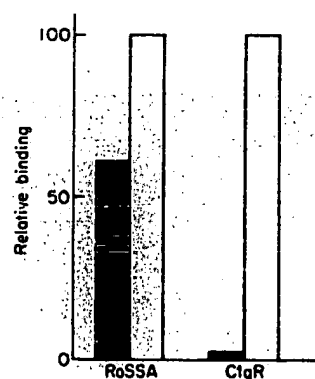


Figure 4. Binding of radioiodinated C1qR or radioiodinated 'RoSSA' to immobilized anti-C1qR antibodies or anti-'RoSSA' antibodies. Radioiodinated C1qR (c.p.m. loaded/well 1×10^6 ; specific activity 9×10^7 c.p.m./ μ g) or radioiodinated RoSSA (component 1 from HPLC (c.p.m. loaded/well 1×10^6 ; specific activity 9×10^7 c.p.m./ μ g) was interacted with immobilized purified antibodies against C1qR (□), 'RoSSA' (■) for 24 hr at 4°. After extensive washing bound c.p.m. were measured, as described in Materials and Methods. Binding is measured relative to the number of c.p.m. bound in the C1qR-anti-C1qR interaction. Results are the average of duplicate experiments. Background binding of radiolabelled ligand to purified anti-ovalbumin-coated wells has been subtracted.

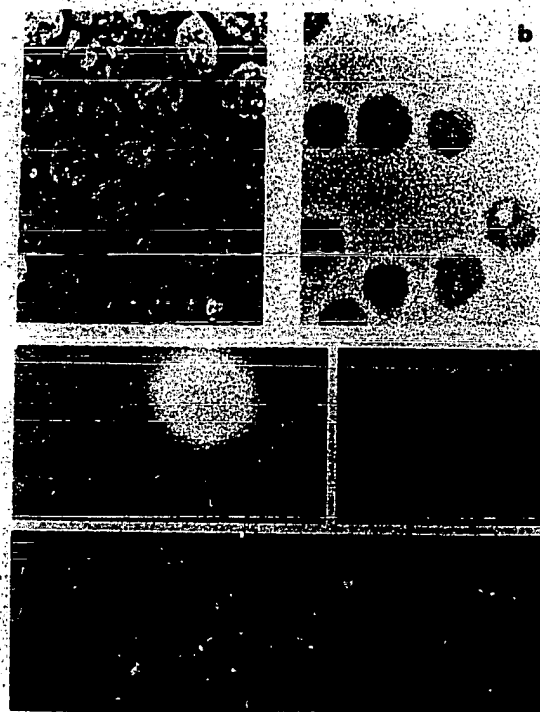


Figure 5. Agglutination of U937 cells by anti-C1qR antibodies. U937 cells (10^7 cells) were incubated with purified anti-C1qR antibodies and control antibodies in PBS containing NaN_3 , as described in Materials and Methods. (a) Agglutination of U937 cells in the presence of purified anti-C1qR antibodies; (b) U937 cells in the presence of control non-immune IgG; (c) immunofluorescence staining of unfixed U937 cells. Unfixed U937 cells were treated with first and second antibody in the presence of NaN_3 at 4°, as described in Materials and Methods. Cells exposed to anti-C1qR antibody showed specific staining of the plasma membrane. Results of several different experiments are shown. The cells treated with control serum were not stained and were not visible in these conditions.

Human ClqR	E P A V Y F K E Q F L D G D G - T S
Murine Calreticulin	D P A I Y F K E Q F L D G D A W T N
Murine B50 melanoma antigen	- - A I Y F K E Q F L N G N A
Human RoSSA/calreticulin	E P A V Y F K E Q F L D G D G W T S
Rat protein 425	- P A - Y F - E Q F L D - R A
Aplysia p407 (Aplysia californica snail neuronal protein 407)	- P T V Y F K E E F G L R A E

Figure 6. Amino-terminal sequence of ClqR and its homology with RoSSA/calreticulin,^{19,20} mouse calreticulin,²² murine B50 melanoma antigen,²³ rat protein 425²⁴ and aplysia protein p407.²⁴ The amino acids not present in the ClqR sequence are underlined.

DISCUSSION

In this paper the partial amino acid sequence of ClqR is described. In Table 1 the sequences of the ClqR peptides generated by different methods are shown. Except for peptides 1, 2 and 3, all the peptides show complete sequence identity with portions of the reported calreticulin²⁰ or RoSSA component sequences.¹⁹ The protein sequence obtained for ClqR indicates that ClqR and the cDNA-defined component^{19,20} are related molecules with a high level of sequence similarity. On a quantitative basis (Table 1) the peptides from ClqR which do not match the RoSSA/calreticulin cDNA-derived sequence have been sequenced at the same quantitative level as the peptides matching the cDNA-derived sequences, and are derived from the same digests. Since ClqR has been purified through multiple steps, ending with a very high resolution ion exchange, this strongly suggests that the non-matching peptides are not from contaminants, and therefore that the sequence of the RoSSA component/calreticulin^{19,20} is not identical throughout to that of ClqR. Further evidence to support this view comes from the composition analysis. McCauliffe *et al.*¹⁹ suggest on the basis of neuraminidase, endo- α -N-acetylgalactosaminidase and glycopeptidase digestion that the RoSSA protein contains no carbohydrate, although the cDNA sequence indicates one potential N-linked glycosylation site. Analysis of ClqR reported earlier,⁶ however, shows that glucosamine is present, and the carbohydrate composition of ClqR strongly suggests that two or three N-linked carbohydrate chains are present on each molecule.⁶ Comparison of amino acid compositions, compiled from the cDNA sequence¹⁹ and experimentally determined for ClqR,⁶ shows considerable similarities, but with significant differences. It is notable that the cysteine content is higher in ClqR (3%) than in RoSSA (1%). Experimental quantitation of cysteine in amino acid analysis generally leads to underestimation, but for ClqR, cysteine was measured, with good agreement, as both an oxidized and an alkylated form.⁶ The cellular localization of the RoSSA antigen component described by McCauliffe *et al.*¹⁹ appears to be intracellular. The data presented in this paper and elsewhere show that ClqR is on the cell surface. Further evidence for non-identity between the two proteins was provided by the antigenic cross-reactivity studies. Anti-ClqR antibodies were found to cross-react with 'RoSSA' (i.e. the species isolated by us from spleen), but

Sjögren's syndrome patients sera with anti-RoSSA antibody showed no reaction with ClqR. The rabbit anti-ClqR antibodies preabsorbed with RoSSA-Sepharose showed positive reaction with ClqR and no reaction with RoSSA. On the basis of cellular localization, composition and antigenicity, therefore, it appears that ClqR is not identical to the protein/cDNA described by McCauliffe *et al.*¹⁹ or Rokeach *et al.*²⁰ but rather is a closely related protein with segments which are identical to those of the calreticulin/RoSSA protein. The presence of extensive identical and non-identical segments in proteins is an unusual type of homology, but as noted by McCauliffe *et al.*,¹⁹ the *Onchocerca volvulus* RAL-1 antigen²¹ shows this type of relationship to the cDNA-derived RoSSA/calreticulin sequence. The *O. volvulus* protein is 63% identical to the RoSSA component over the region of sequence available for comparison, but contains segments of completely identical, and segments of completely different sequence. A group of proteins, many of which are characterized as calcium-binding proteins, have a similar N-terminal sequence to ClqR and to calreticulin/RoSSA component. Some of these are shown in Fig. 6. The complete sequence of the human calreticulin/RoSSA component is very closely related (92% identity) to the sequences of mouse and rabbit calreticulin,²² as might be expected if it is a species homologue. Among the proteins shown in Fig. 6, it is of interest that two mouse proteins, melanoma antigen B50 and calreticulin, have very similar but not identical N-terminal sequences, but the reported cellular localization of the two molecules is different. B50 antigen has been reported on the cell surface of non-melanoma and melanoma cell lines,²³ whereas calreticulin has been reported as an endoplasmic protein.²² The relationship between two mouse proteins may resemble that between the two human proteins, ClqR and the calreticulin/RoSSA species.

The interpretation of the relationship of ClqR to the reported RoSSA¹⁹ or calreticulin²⁰ cDNA sequence is made complex by controversy as to whether the cDNA sequence does indeed represent a RoSSA component, or whether it represents calreticulin. Calreticulin is not thought to be recognized by autoantibodies in Sjögren's syndrome sera.²⁰ However in this study, we have isolated by high resolution chromatographic methods a homogeneous protein species from spleen which has the same N-terminal sequence as the reported RoSSA component,^{18,19} and is recognized by antibodies in Sjögren's syndrome

sera which have been characterized by standard methods as RoSSA positive. To this extent, our results are in agreement with McCauliffe *et al.*¹⁹ We have also isolated a homogeneous protein, C1qR with the same N-terminal sequence, which is not recognized by Sjögren's syndrome antibodies, which has internal sequence which is different from the cDNA-derived RoSSA or calreticulin sequence. The discussion in ref. 19 on the similarity between RAL-I antigen and the RoSSA/calreticulin sequence, and the data presented in Fig. 6, suggest that there is a group of very closely related proteins, which exhibit an unusual type of homology, with blocks of identical sequence, and blocks of completely unrelated sequence. If this is the case, then the use of cDNA sequencing, particularly polymerase chain reaction (PCR)-based cDNA, without extensive use of direct confirmation by protein sequence will not serve to distinguish between closely related proteins.

In conclusion, the similarity in polypeptide size, overall charge and in blocks of amino acid sequence between C1qR, the reported human RoSSA component, calreticulin, *O. volvulus* RAL-I antigen, murine B50, aplysia p407 and rat 0425, indicate that C1qR is part of a highly conserved protein family.

ACKNOWLEDGMENTS

We thank Miss B. Moffatt and Mrs J. U. Newell for technical assistance. We also thank Dr K. B. M. Reid for useful discussion and advice. This project was funded by the British Lung Foundation and the Colt Foundation. R.M. is a British Lung Foundation Fellow.

REFERENCES

- DICKLER H.B. & KUNKEL H.G. (1972) Interaction of aggregated γ -globulin with B-lymphocytes. *J. exp. Med.* **136**, 191.
- TENNER A. (1989) C1q interaction with cell surface receptors. *Behring Inst. Mitt.* **84**, 220.
- GHEBREHIWET B. (1989) Functions associated with the C1q receptor. *Behring Inst. Mitt.* **84**, 204.
- ERDEI A. & REID K.B.M. (1988) Characterization of C1q binding material released from the membrane of Raji and U937 cells by limited proteolysis with trypsin. *Biochem. J.* **255**, 493.
- MALHOTRA R., REID K.B.M. & SIM R.B. (1988) Studies on the isolation of human C1q-receptor. *Biochem. Soc. Trans.* **16**, 735.
- MALHOTRA R. & SIM R.B. (1989) Chemical and hydrodynamic characterisation of the human leucocyte receptor for complement subcomponent C1q. *Biochem. J.* **262**, 625.
- MALHOTRA R., THIEL S., REID K.B.M. & SIM R.B. (1990) Human leukocyte C1q receptor binds other soluble proteins with collagen domains. *J. exp. Med.* **172**, 955.
- THIEL S. & REID K.B.M. (1989) Structure and functions associated with the group of mammalian lectins containing collagen-like sequences. *FEBS Lett.* **250**, 78.
- MALHOTRA R., HAURUM J., THIEL S. & SIM R.B. (1992) Interaction of C1q Receptor (C1qR) with lung surfactant protein-A (SP-A). *Eur. J. Immunol.* **22**, 1437.
- FRAKER P.J. & SPECK J.C., JR. (1978) Protein and cell membrane iodination with a sparingly soluble chloroamide 1,3,4,6-tetra chloro-3a,6a diphenylglycoluril. *Biochem. biophys. Res. Commun.* **80**, 849.
- WU D.-H., TAVONI A., GARZELLI C., NERI R., VITALI C. & BOMBARDIERI S. (1989) A simple method for the biochemical purification of Ro/SSA antigen. *J. immunol. Meth.* **121**, 219.
- VENABLES P.J., SMITH P.R. & MAINI R.N. (1983) Purification and characterisation of Sjögren's syndrome A and B antigens. *Clin. exp. Immunol.* **54**, 731.
- LAEMMLI U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **316**, 72.
- FAIRBANKS G., STECK T.L. & WALLACH D.F.H. (1971) Electrophoretic analysis of the major polypeptide of the human erythrocyte membrane. *Biochemistry*, **10**, 2606.
- PRAHL J.W. & PORTER R.R. (1968) Allotype-related sequence variation of the heavy chain of rabbit immunoglobulin G. *Biochem. J.* **107**, 753.
- KÖLBLE K., RUKAVINA V. & KALDEN J.R. (1987) High resolution electrophoresis for the allotyping of human C4. *J. immunol. Meth.* **96**, 69.
- MALHOTRA V. & SIM R.B. (1985) Expression of complement factor H on the cell surface of the human monocytic cell line U937. *Eur. J. Immunol.* **15**, 935.
- LIEU T.S., NEWKIRK M.M., CAPRA J.D. & SONTHEIMER R.D. (1988) Molecular characterisation of human Ro/SSA antigen. *J. clin. Invest.* **82**, 96.
- MCCAULIFFE D.P., LUX F.A., LIEU T.S., SANZ L., HANKE J., NEWKIRK M.M., BACHINSKI L.L. *et al.* (1990) Molecular cloning, expression, and chromosome 19 localization of human Ro/SSA autoantigen. *J. clin. Invest.* **85**, 1379.
- ROKEACH L.A., HASELBY J.A., MELOF J.F., SMEENK R.J.T., UNNASCH T.R., GREENE B.M. & HOCH S.O. (1991) Characterisation of the autoantigen-calreticulin. *J. Immunol.* **147**, 3031.
- UNNASCH T.R., GALLIN M.Y., SOBOSLAY P.T., ERTTMANN K.D. & GREEN B.M. (1988) Isolation and characterisation of expression cDNA clones encoding antigens of *Onchocerca volvulus* infective larvae. *J. clin. Invest.* **82**, 262.
- SMITH M.J. & KOCH L.E. (1989) Multiple zones in the sequence of calreticulin (CRP 55, calregulin, HACBP), a major calcium binding ER/SR protein. *EMBO J.* **8**, 3581.
- GERSTEN D.M., BUWAARD K.E., LAW L.W. & HEARING V.J. (1991) Homology of the B50 murine melanoma antigen to the Ro/SSA antigen of human systemic lupus erythematosus and calcium binding proteins. *Biochem. biophys. Acta*, **1096**, 20.
- KENNEDY T.E., GAWINOWICZ M.A., BARZILAI A., KANDEL E.R. & SWEATT J.D. (1988) Sequencing of proteins from two-dimensional gels by using in situ digestion and transfer of peptides to polyvinylidene difluoride membranes: application to proteins associated with sensitization in Aplysia. *Proc. natl. Acad. Sci. U.S.A.* **85**, 7008.

STIC-ILL

Adams

From: Romeo, David
Sent: Monday, June 04, 2001 12:05 PM
To: STIC-ILL
Subject: 09503421 Stuart GR, Lynch NJ, Day AJ, Schwaeble WJ, Sim RB.

20-

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... -305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE
Stuart GR, Lynch NJ, Day AJ, Schwaeble WJ, Sim RB.

The Clq and collectin binding site within Clq receptor (cell surface calreticulin).

Immunopharmacology. 1997 Dec;38(1-2):73-80.

PMID: 9476117

ADONIS - Electronic Journal Services

Requested by

Adonis

Article title The Clq and collectin binding site within Clq receptor (cell surface calreticulin)

Article identifier 0162310998002945

Authors Stuart_G_R Lynch_N_J Day_A_J Schwaeble_W_J Sim_R_B

Journal title Immunopharmacology

ISSN 0162-3109

Publisher Elsevier Netherlands

Year of publication 1997

Volume 38

Issue 1-2

Supplement 0

Page range 73-80

Number of pages 8

User name Adonis

Cost centre

PCC \$20.00

Date and time Tuesday, June 05, 2001 3:27:36 AM

Copyright © 1991-1999 ADONIS and/or licensors.

The use of this system and its contents is restricted to the terms and conditions laid down in the Journal Delivery and User Agreement. Whilst the information contained on each CD-ROM has been obtained from sources believed to be reliable, no liability shall attach to ADONIS or the publisher in respect of any of its contents or in respect of any use of the system.



The C1q and collectin binding site within C1q receptor (cell surface calreticulin)¹

Guy R. Stuart^{a,*}, Nicholas J. Lynch^b, Anthony J. Day^c, Wilhelm J. Schwaeble^b,
Robert B. Sim^a

^a MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

^b Department of Microbiology and Immunology, School of Medicine, University of Leicester, University Road, Leicester LE1 9HN, UK

^c Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Received 7 May 1997; accepted 20 July 1997

Abstract

C1q receptor (C1qR/collectin receptor/cC1qR) has an almost complete amino acid sequence identity with calreticulin (CRT). C1qR/CRT is located on the surface of many cell types. Binding of C1q to C1q receptor elicits a range of immunological responses. C1qR also interacts with the collectins SP-A, MBL, CL43 and conglutinin via a cluster of charged residues on the collagen tails of the ligands. In order to localise C1q and collectin binding activity within C1qR/CRT, recombinant C1qR/CRT domains [N (residues 18–196), P (197–308) and C (309–417)] were produced. Both the N- and P-domains bound to C1q, demonstrating that the binding site spans the intersection of these domains. Amino acid alignment analysis identified a putative CUB module within this region. This S-domain (residues 160–283) was expressed and showed concentration-dependent binding to immobilised C1q, demonstrating that it contains the C1q binding site. Competitive inhibition studies of the S-domain–C1q interaction revealed that the S-domain binds to C1q collagen tails and to the collectin proteins, SP-A, MBL, CL43 and conglutinin. The C1q and collectin binding site on C1qR/CRT has therefore been localised to the S-domain. © 1997 Elsevier Science B.V.

Keywords: C1q receptor; Calreticulin; C1q; Collectin; Complement

1. Introduction

C1q receptor (cC1qR or collectin receptor) was the first cell surface receptor for the recognition subunit of the first component of complement to be isolated (Ghebrehiwet et al., 1984). C1qR has an almost identical amino acid sequence to calreticulin (CRT) (Malhotra et al., 1993a), a highly conserved abundant multifunctional calcium binding protein, indicating that it is a cell-surface expressed isoform of CRT. C1qR and CRT are both 47 kDa proteins (from mass spectrometry (Malhotra and Sim, 1993))

Abbreviations: C1qR, C1q receptor; CRT, calreticulin; SP-A, lung surfactant protein A; MBL, mannose binding lectin; ER, endoplasmic reticulum; SLE, systemic lupus erythematosus; PDI, protein disulphide isomerase

* Corresponding author. Fax: +44-1865-275729.

¹ It is becoming increasingly clear that the protein variously denoted as 'C1qR', 'cC1qR' or 'Collectin receptor' is a cell-surface isoform of calreticulin. Calreticulin has itself been denoted as CRT, CaR or CR (and mistakenly as the Ro-SSA antigen due to its possible association with the Ro complex). CRT is now commonly used as the symbol for calreticulin to avoid confusion with CR, a generic term for complement receptors.

with an apparent molecular weight of 56 kDa on SDS-PAGE. C1qR is located on the surface of leucocytes, endothelial cells, fibroblasts, platelets and specialised epithelia (Tenner, 1993; Malhotra and Sim, 1993). Binding of C1q to C1qR on these cell types elicits a range of immunological responses, such as phagocytosis, enhanced cytokine and antibody production, and antibody-dependent cell cytotoxicity (Ghebrehwet and Peerschke, 1993; Malhotra, 1993b). C1qR also binds to the collectins lung surfactant protein-A (SP-A), mannose binding lectin (MBL), CL43 and conglutinin (Malhotra et al., 1990, 1992), resulting in enhanced phagocytosis of collectin-bearing particles via C1qR-bearing cells (Geertsma et al., 1994).

CRT has a highly diverse functional repertoire. CRT contains a C-terminal KDEL endoplasmic reticulum retention sequence (Fliegel et al., 1989), yet CRT forms have been identified in many diverse cellular compartments, including the cell surface. Cell surface CRT may trigger cell spreading (White et al., 1995) and mediate the mitogenic effects of fibrinogen (Gray et al., 1995). Physiologically, CRT is involved in regulation of intracellular Ca^{2+} homeostasis (Krause, 1996; Michalak, 1996) acting as an ER molecular chaperon due to its lectin characteristics (reviewed in Helenius et al., 1997) and the regulation steroid-sensitive gene expression (Burns et al., 1994; Dedhar et al., 1994). Recent studies with CRT knock-outs have demonstrated that CRT is absolutely essential for integrin-mediated calcium signalling and cell adhesion (Coppolini et al., 1997). The role of CRT in autoimmunity, where it may participate in SLE, is being investigated (Eggleton et al., 1997). CRT, like C1q receptor, binds to C1q and the collectins (Eggleton et al., 1994; Stuart et al., 1996a).

The C1q–C1qR interaction is ionic-strength-dependent (Arvieux et al., 1984), and involves a region of charged residues on the C1q collagen stalks (Malhotra et al., 1993c). CRT and C1qR interact in an identical fashion with C1q and collagens (Stuart et al., 1996a), indicating that there is an homologous C1q binding region within C1qR and CRT.

Recombinant CRT domains to be tested for C1q and collectin binding function were produced from a cDNA clone (pHCRT-1) isolated from a human umbilical vein endothelial cell cDNA library (Stuart et

al., 1996a). These domains, as described below, are based upon structural predictions for the molecule and have previously been used to localise CRT function within the molecule. The amino-terminal N-domain contains the binding regions for PDI (Baksh et al., 1995a), Zn^{2+} (Baksh et al., 1995b) and integrins (Leung-Hagesteijn et al., 1994). The proline-rich central P-domain contains the high affinity Ca^{2+} binding site (Baksh and Michalak, 1991) and the lectin site (D. Williams, cited in Krause and Michalak, 1997) within two sets of highly conserved repeats. The acidic C-domain contains the ER retention terminal KDEL signal (McCauliffe et al., 1990) and the low affinity Ca^{2+} binding site (Baksh and Michalak, 1991). Our previous studies have indicated that the C1q binding site lies across the intersection of the N- and P-domains (Stuart et al., 1996a). Within this region we have identified and expressed a 123 amino acid region containing a putative C1r/C1s (also termed CUB) module (Day et al., 1993) based upon amino acid sequence alignments. We termed this segment the S-domain and show here that it contains the C1q and collectin binding site of C1qR/CRT.

2. Materials and methods

2.1. Purification and radioiodination of C1qR, C1q and collectins

Native C1qR was purified from human U937 cells as previously described (Malhotra et al., 1993a). C1qR and S-domain samples were iodinated by the iodogen method (Fraker and Speck, 1978). C1q was purified as previously described (Reid, 1981) and radioiodinated as described by Bolton and Hunter (1973) as this method of iodination causes less damage to large, oxidation-sensitive molecules such as C1q than the more frequently utilised iodogen method (Stuart et al., 1996b). Radiolabelled proteins were stored at 4°C. C1q collagen tails were prepared by T. Gascoyne as described by Reid (1976). Collectins were purified as previously described (Malhotra et al., 1990; Holmskov et al., 1995). Rabbit F_c was prepared from rabbit IgG by papain digestion and cation exchange chromatography, as described by Johnstone and Thorpe (1987).

2.2. Prokaryotic expression of recombinant calreticulin domains

Given that CRT, Clr and Cls all interact with Clq, a sequence comparison was performed to investigate the structural basis for this interaction. A region that may correspond to a CUB module was identified in CRT and was analyzed by multiple sequence alignment as described previously (Day et al., 1993). This region, termed the S region (Cls-like (CUB) domain), spans the intersection of the N- and P-domains (residues 160–283).

A 1.9-kb cDNA clone for CRT (phCRT-1) was isolated from a human umbilical vein endothelial cell library (kindly donated by B. Seed, MIT, Boston, MA) in the eukaryotic expression vector CDM8 (Aruffo and Seed, 1987). Sequence analysis revealed that phCRT-1 comprised the complete coding sequence for CRT with absolute identity to the previously published human CRT sequence (McCauliffe et al., 1990).

The thiobond expression system was used to produce N-, P-, C- and S-domains of ClqR/CRT (representing the N-terminal region, the proline-rich central region, the C-terminal region, and a region spanning the intersection of the N- and P-domains (as described above) (Fig. 1). The individual domains were expressed as thioredoxin fusion proteins in *E. coli* using the plasmid pTrxfus (Invitrogen B.V., Leek, The Netherlands) as described previously (Stuart et al., 1996a).

Samples were assayed for recombinant calreticulin domain expression by SDS-PAGE (Laemmli, 1970) and by Western blotting with rabbit antisera to: (1) whole ClqR (raised against human ClqR purified from U937 cells (Malhotra et al., 1993a)), (2) CRT C-terminal region (raised against a GST fusion protein containing the final 18 residues of recombinant human CRT), and (3) CRT N-terminal region (raised against a GST fusion protein containing residues 7–18 of recombinant human CRT).

2.2.1. Interaction of immobilised recombinant human ClqR/CRT domains with radiolabelled Clq

Binding experiments with the ClqR/CRT domains were performed throughout in low salt (10 mM potassium phosphate, 0.5 mM EDTA (pH 7.4)) in order to maximise the ionic interaction with Clq.

Microtitre plates were coated with the N-, P-, C-

N-DOMAIN	
1	MLAVVLLLLLLHAYV E PAVYFKEQFLDGGG
33	WTSRWIESKHKSDFGKFLSSGKPYGDEEKDK
65	GLQTSQDARFYALSASFEPFSKKGCTLVVCF
97	VXHEQNIDCGGGYVRLFPKSLCCTDMKGGSEY
129	NIMFGPDICGPGTKKKVHVIFNYKGNVL:HXD
161	<u>IRCKDDEFTHLVTL:VRPQNTVVRVIDNSQWE</u>
193	<u>SGSL</u>
P-DOMAIN	
197	<u>EDDMDF:PPKKIKDPDASKPEDWDERAKICCP</u>
229	<u>TDSPKPEDWDXPEHIPPDAKVPEDWDEEMCGE</u>
261	<u>NEPPVIONPEYKGFHKPQ:DNPDYKGTW:HP</u>
293	EIDNPEYSPDPSIYAY
C-DOMAIN	
309	DNFGVLGLDLWQVKSCTIFDNFL:TNDEAYAE
341	EFGNETMGVTKAAEKQMKDXQCEECRLKEEEE
373	DKKRKEEEEAEDCKEDDEDKDEDEDEDECKRED
405	EEDDVPGQAKDEL

Fig. 1. Amino acid sequence of CRT. Amino acid sequences were deduced from the nucleotide sequence of human CRT (McCauliffe et al., 1990). The signal sequence residues are shown in small font. The N-, P- and C- domains are indicated. The S-domain is underlined. Domain constructs were expressed as thioredoxin fusion products.

and S-domains and with three controls, ClqR, BSA and thioredoxin (8 μ g/ml in 35 mM NaHCO₃, 15 mM Na₂CO₃ pH 9.6) for 2 h at 37°C. Non-specific interactions were blocked by incubation with 10 mM potassium phosphate, 0.5 mM EDTA pH 7.4 containing BSA (10 mg/ml). Any free -SH groups in the samples, due to the presence of the thioredoxin fusion protein, were blocked by a brief washing step using the phosphate buffer containing 2 mM iodoacetamide. After washing, serial dilutions of radioiodinated Clq (in 10 mM potassium phosphate, 0.5 mM EDTA, pH 7.4) were added to the wells and incubated for 2 h at 37°C. Wells were washed three times with phosphate buffer and bound radioactivity eluted with 100 μ l 4M NaOH and measured.

2.2.2. Concentration-dependent binding of radiolabelled S-domain to immobilised Clq

Clq binds to the F_c regions of IgG. This property was utilised in order to correctly orient the Clq on microtitre plates. Breakable microtitre plates (Life Sciences International (UK) Ltd, Basingstoke,

Hampshire, UK) were coated with rabbit F_c (5 μ g per well in 35 mM NaHCO_3 , 15 mM Na_2CO_3 pH 9.6). Non-specific sites were blocked as described above, and the wells were incubated with C1q (0.8 μ g per well in 10 mM potassium phosphate, 0.5 mM EDTA, pH 7.4). Certain wells were also coated with BSA (0.8 μ g per well) as a negative control. After further washing, serial dilutions of radioiodinated S-domain (in 10 mM potassium phosphate, 0.5 mM EDTA, pH 7.4) were added to the wells and incubated for 2 h at 37°C. Wells were washed three times with the phosphate buffer and bound radioactivity in the individual wells measured.

2.2.3. Competitive inhibition of the S-domain–C1q interaction by fluid phase C1q, collectins and C1q collagen tails

C1q was immobilised onto F_c -coated microtitre plates as described above. Non-specific binding was blocked by incubation with 10mM potassium phosphate, 0.5mM EDTA (pH 7.4) containing BSA (10 mg/ml). Serial dilutions of the collectins (SP-A, MBL, SP-D, CL43), C1q, C1q tails and BSA (maximum quantity = 9 μ g/well) were prepared in 10 mM potassium phosphate, 0.5 mM EDTA (pH 7.4). Each dilution (100 μ l) was then incubated for 1 h at 37°C with a constant level of radiolabelled S-domain and loaded onto the plate. Following 2 h incubation at 37°C, wells were extensively washed and bound radioactivity measured.

3. Results

3.1. Amino acid sequence alignments

A region spanning the intersection of the N- and P-domains of CRT has previously been implicated in C1q binding (Stuart et al., 1996a). Amino acid sequence alignment showed that a region within CRT (residues 160–283) may correspond to a CUB module. Two CUB modules, together with an EGF module, form a binding region within Clr_2Cls_2 for the collagenous tails of C1q. C1qR competes with Clr_2Cls_2 for binding to C1q, implying a similarity in C1q binding sites on Clr, Cls and C1qR (Sobel and Bokisch, 1975; Van den Berg et al., 1995). Therefore this segment, the S-domain (residues 160–283, see Fig. 1), was tested for C1q binding.

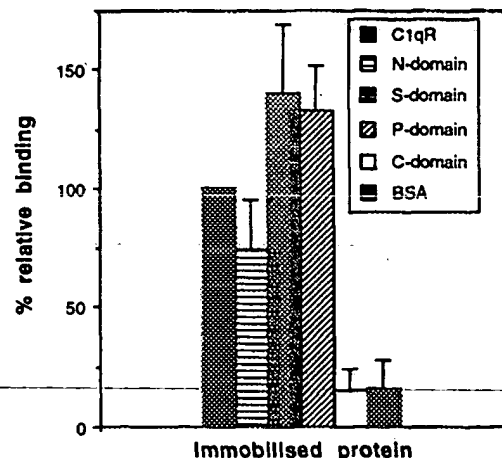


Fig. 2. Binding of C1q to calreticulin domains. Solid-phase bound domains, with appropriate controls, were incubated with radioiodinated C1q. Binding levels of four separate experiments, at saturation, are shown, calculated as % (bound/loaded). These percentages are then standardised against the results for C1qR.

3.2. Interaction of recombinant human calreticulin domains with C1q

N-, P-, C-, and S-domains of human CRT were expressed as thioredoxin fusion proteins. Correct expression was verified by SDS-PAGE and Western blotting. Fig. 2 summarises the results of four sepa-

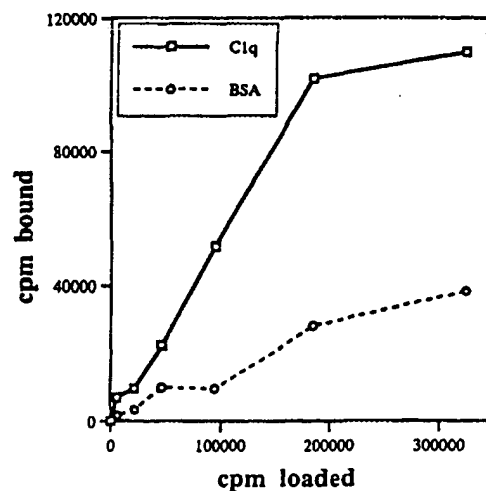


Fig. 3. Binding of S-domain to immobilised C1q. Serial dilutions of radiolabelled S-domain were bound to immobilised C1q and BSA. After extensive washing, bound radioactivity was measured as described in Section 2. The mean values of three separate binding assays are shown.

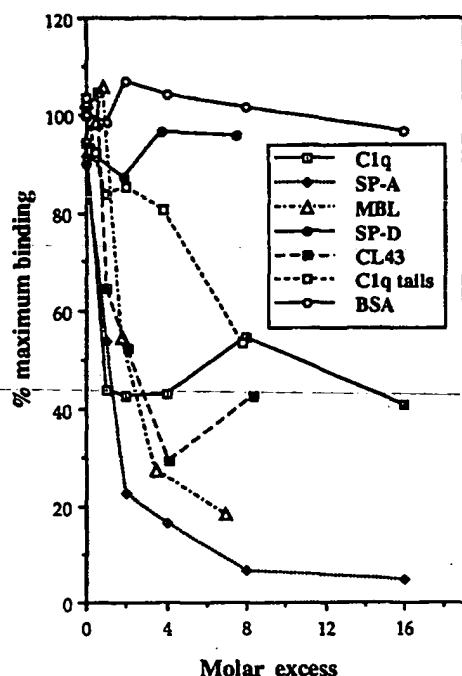


Fig. 4. Inhibition of S-domain-C1q interaction by collectins, C1q and C1q collagen tails. Constant levels of radiolabelled S-domain were pre-incubated with serial dilutions of unlabelled C1q, C1q tails and collectin proteins. The incubation mixture was bound to, and eluted from, solid phase C1q. Mean values of three separate inhibition assays are shown.

rate solid phase direct binding experiments. Significant binding to radioiodinated C1q was observed for C1qR, the S-domain, the P-domain, and, to a lesser extent, the N-domain. The C-domain showed no binding.

Serial dilutions of radioiodinated S-domain were incubated with immobilised C1q and BSA (Fig. 3). Concentration-dependent, saturable binding was observed to C1q but not to BSA.

3.3. Competitive inhibition of C1q-S-domain interaction by C1q tails and collectins

C1q was immobilised on microtitre plates by interaction with solid phase F_c . Fig. 4 shows the results of three separate competitive inhibition studies of the S-domain-C1q interaction. As expected, native fluid-phase C1q demonstrates concentration-dependent inhibition. C1q tails also cause inhibition, indicating that the interaction of the S-domain with

C1q is via the collagenous C1q tails. Inhibition studies with the collectin proteins demonstrated that SP-A, MBL and CL43 interact with the S-domain, via the same, or an overlapping binding site as C1q. SP-D and BSA did not inhibit the S-domain-C1q interaction.

4. Discussion

The 56 kDa receptor for C1q, MBL, CL43 and SP-A is present on the surface of many cell types. C1qR has an amino acid sequence almost identical to calreticulin. Although CRT contains an endoplasmic reticulum retention and retrieval sequence, cell surface forms of CRT have been identified, indicating that C1qR is a membrane-associated isoform of calreticulin. Studies have demonstrated the existence of different molecular weight forms of CRT in the ER and the plasma membrane (Zhu et al., 1997). At present it remains unclear what property of cell surface CRT enables it to escape the KDEL receptor in the ER and presents itself on the cell surface. Unlike a recently identified additional 126 kDa receptor for C1q (Guan et al., 1994; Nepumuceno et al., 1997), CRT does not contain a membrane-spanning domain and its mode of attachment to the membrane remains unclear.

We have previously demonstrated that the C1q binding site within CRT, and therefore within C1qR, lies across the intersection of the N- and P-domains (Stuart et al., 1996a). C1qR and C1r₂C1s₂ cross-compete for binding to C1q, indicating that they have overlapping binding sites on C1q (Sobel and Bokisch, 1975; Van den Berg et al., 1995). Consequently, C1qR/CRT and C1r-C1s protein sequences were compared. A region of CRT (residues 160–283) was identified that may correspond to a CUB module. This is of particular interest as two CUB modules, together with an EGF module, form the globular region at the end of the C1r or C1s 'dumbbell' and this region mediates the interaction of C1r and C1s with each other and with C1q, via the collagenous region of C1q (Day et al., 1993; Arlaud et al., 1993). This S-domain shows very high cross-species conservation. Furthermore, the S-domain spans the N- and P-domains, previously implicated in C1q binding (Stuart et al., 1996a).

The binding of the C1q tails to the S-domain confirms previous observations that C1q interacts with C1qR via its collagenous stalk region. The S-domain–C1q interaction was found to be ionic-strength-dependent (results not shown) indicating that it is predominantly a protein–protein interaction based upon a region of charged residues on the collagen stalks of the C1q ligand (Malhotra et al., 1993c). However, it is possible that there may also be a protein–carbohydrate interaction. CRT utilises its lectin characteristics as a chaperon (Spiro et al., 1996; Helenius et al., 1997) and in cell adhesion (White et al., 1995). Furthermore, the spermadhesin CUB module binds to carbohydrate (Day et al., 1993). Using deletion mutants of CRT, the lectin site in CRT has been localised to the three sets of repeats A (PxxIxDPDAxKPEDWDE: residues 204–220; 221–237; 238–254) and B (GxWxPPxIxNPxYx: residues 258–271; 272–285; 286–299) that lie within the highly charged first part of the P-domain of CRT. Truncation of even a portion of the repeats abolished glycoprotein binding to CRT (D. Williams, cited by Krause and Michalak, 1997). The S-domain contains repeats A and part of repeats B. It is possible that the lectin characteristic of this region may supplement the predominantly ionic protein–protein interaction. Within the ER, CRT may function as a molecular chaperon by binding transiently to monoglucosylated folding intermediate glycoproteins. The minimum structural requirement Glc₁Man₃GlcNAc is essential for CRT binding: no interaction with Glc₁Man₄GlcNAc was observed (Spiro et al., 1996). The collagen stalks of C1q contain 69% of the total C1q carbohydrate, present as glucosylgalactosyl disaccharide units (Reid, 1979). These oligosaccharides are unlikely to function as core binding elements although they may be able to supplement the more important protein–protein interaction. The collagen stalks of the collectins are likely to be glycosylated similarly to C1q, although this has not been investigated in detail.

All the collectins, with the probable exception of SP-D, bind the C1q receptor (Malhotra et al., 1990). We have demonstrated that MBL, SP-A and CL43 all bind to the S-domain of CRT. SP-D did not bind to the S-domain. These results are consistent with previous observations with native C1qR (Malhotra et al., 1990, 1992). Comparison of the N-terminal re-

gions of the collagenous regions of C1q, MBL, CL43, SP-A and conglutinin led to the identification of a possible C1qR binding site, composed of five collagen repeats (Gly–X–Y triplets) with many charged residues in the X and Y positions (Malhotra et al., 1993c). SP-D contains a similar site but apparently binds to a different receptor on alveolar macrophages (Miyamura et al., 1994).

Acknowledgements

This work was supported by the Arthritis and Rheumatism Council (grants SO534 and SO113). G.R.S. is an MRC student at the Oxford Centre for Molecular Sciences.

References

- Arlaud, G.J., Thielens, N.M., Illy, C., 1993. Assembly of the C1 complex. *Behring Inst. Mitt.* 93, 189–195.
- Aruffo, A., Seed, B., 1987. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA* 84, 8573–8577.
- Arvieux, J., Reboul, A., Bensa, J.C., Colomb, M.G., 1984. Characterisation of the C1q receptor on a human macrophage cell line. U937. *Biochem. J.* 218, 547–555.
- Baksh, S., Michalak, M., 1991. Expression of calreticulin in *E. coli* and identification of its calcium binding domains. *J. Biol. Chem.* 266, 21458–21465.
- Baksh, S., Burns, K., Andrin, C., Michalak, M., 1995a. Interaction of calreticulin with protein disulfide isomerase. *J. Biol. Chem.* 270, 31338–31344.
- Baksh, S., Spamer, C., Heilmann, C., Michalak, M., 1995b. Identification of the Zn²⁺ binding region in calreticulin. *FEBS Lett.* 376, 53–57.
- Bolton, A.E., Hunter, W.M., 1973. The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. *Biochem. J.* 133, 529–539.
- Burns, K., Duggan, B., Atkinson, E.A., Famulski, K.S., Nemer, M., Bleackley, R.C., Michalak, M., 1994. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* 367, 476–480.
- Coppolini, M.G., Woodside, M.J., Demareux, N., Grinstein, S., St-Arnaud, R., Dedhar, S., 1997. Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* 386, 843–847.
- Day, A.J., Barlow, P.N., Steinkasserer, A., Campbell, I.D., Sim, R.B., 1993. Progress in determining module structures in C1r and C1s. *Behring Inst. Mitt.* 93, 31–40.
- Dedhar, S., Rennie, P.S., Shago, M., Leung-Hagesteijn, C.Y., Yang, H., Filmus, J., Hawley, R.G., Bruchovsky, N., Cheng,

- H., Matusik, R.J., Giguere, V., 1994. Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* 367, 480–483.
- Eggleton, P., Lieu, T.S., Zappi, E.G., Sastry, K., Coburn, J., Zaner, K.S., Sontheimer, R.D., Capra, J.D., Ghebrehwet, B., Tauber, A.I., 1994. Calreticulin is released from activated neutrophils and binds to C1q and mannan-binding protein. *Clin. Immunol. Immunopathol.* 72, 405–409.
- Eggleton, P., Kishore, U., Reid, K.B.M., Sontheimer, R.D., 1997. Clinical relevance of calreticulin in systemic lupus erythematosus. *Lupus* 6, 564–571.
- Fliegel, L., Burns, K., MacLennan, D.H., Reithmeier, R.A.F., Michalak, M., 1989. Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 264, 21522–21528.
- Fraker, P.J., Speck, J.C. Jr., 1978. Protein and cell membrane iodination with sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80, 849–857.
- Geertsma, M.F., Nibbering, P.H., Haagsman, H.P., Daha, M.R., Van Furth, R., 1994. Binding of surfactant protein A to C1q receptors mediates phagocytosis of *Staphylococcus aureus* by monocytes. *Am. J. Physiol.* 267, L578–L584.
- Ghebrehwet, B., Silvestri, L., McDevitt, C., 1984. Identification of the Raji cell membrane-derived C1q inhibitor as a receptor for human C1q. *J. Exp. Med.* 160, 1375–1389.
- Ghebrehwet, B., Peerschke, E.I.B., 1993. The C1qR participates in immunoregulation and signal transduction. *Behring Inst. Mitt.* 93, 236–240.
- Gray, A.J., Park, P.W., Broekelmann, T.J., Laurent, G.J., Reeves, J.T., Stenmark, K.R., Mecham, R.P., 1995. The mitogenic effects of the B beta chain of fibrinogen are mediated through cell surface calreticulin. *J. Biol. Chem.* 270, 26602–26606.
- Guan, E., Robinson, S.L., Goodman, E.B., Tenner, A.J., 1994. Cell-surface protein identified on phagocytic cells modulates the C1q-mediated enhancement of phagocytosis. *J. Immunol.* 152, 4005–4018.
- Helenius, A., Trombetta, E.S., Hebert, D.N., Simons, J.F., 1997. Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biol.* 7, 193–200.
- Holmskov, U., Laursen, S.B., Malhotra, R., Wiedemann, H., Timpl, R., Stuart, G.R., Tornøe, I., Madsen, P.S., Reid, K.B.M., Jensenius, J.-C., 1995. Comparative study of the structural and functional properties of a bovine plasma C-type lectin, collectin-43, with other collectins. *Biochem. J.* 305, 889–896.
- Johnstone, A., Thorpe, R., 1987. *Immunochemistry in Practice*, 2nd ed. Blackwell Scientific.
- Krause, K.H., 1996. Calreticulin and calcium storage. In: Michalak, M. (Ed.), *Calreticulin*. R.G. Landes Co., Austin, TX, pp. 59–71.
- Krause, K.H., Michalak, M., 1997. Calreticulin. *Cell* 88 (4), 439–443.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Leung-Hagesteijn, C.Y., Milankov, K., Michalak, M., Wilkins, J., Dedhar, S., 1994. Cell attachment to extracellular matrix substrates is inhibited upon downregulation of expression of calreticulin, an intracellular integrin alpha-subunit-binding protein. *J. Cell Sci.* 107, 589–600.
- McCaulliffe, D.P., Lux, F.A., Lieu, T.-S., Sanz, I., Hanke, J., Newkirk, M.M., Bachinski, L.L., Itoh, Y., Siciliano, M.J., Reichlin, M., Sontheimer, R.D., Capra, J.D., 1990. Molecular cloning, expression, and chromosome 19 localisation of a human Ro/SSA autoantigen. *J. Clin. Invest.* 85, 1379–1391.
- Malhotra, R., 1993b. Collectin receptor: structure and function. *Behring Inst. Mitt.* 93, 254–261.
- Malhotra, R., Thiel, S., Reid, K.B.M., Sim, R.B., 1990. Human leukocyte C1q receptor binds other soluble proteins with collagen domains. *J. Exp. Med.* 172, 955–959.
- Malhotra, R., Haurum, J., Thiel, S., Sim, R.B., 1992. Interaction of C1q receptor with lung surfactant protein A. *Eur. J. Immunol.* 22, 1437–1445.
- Malhotra, R., Willis, A.C., Jensenius, J.C., Jackson, J., Sim, R.B., 1993a. Structure and homology of human C1q receptor (collectin receptor). *Immunology* 78, 341–348.
- Malhotra, R., Laursen, S., Willis, A.C., Sim, R.B., 1993c. Localisation of the receptor binding site in the collectin family of proteins. *Biochem. J.* 293, 15–19.
- Malhotra, R., Sim, R.B., 1993. Collectin receptor. In: Haerberli, A. (Ed.), *Human Protein Data*. VCH, Weinheim.
- Michalak, M. (Ed.), 1996. *Calreticulin*. R.G. Landes Co., Austin, TX.
- Miyamura, K., Leigh, L.E.A., Lu, J., Hopkin, J., Lopez-Bernal, A., Reid, K.B.M., 1994. Surfactant protein D binding to alveolar macrophages. *Biochem. J.* 300, 237–242.
- Nepumuceno, R.R., Henshen-Edman, A.H., Burgess, W.H., Tenner, A.J., 1997. cDNA cloning and primary structure analysis of C1qR_p, the human C1q/MBL/SPA receptor that mediates enhanced phagocytosis in vitro. *Immunity* 6, 115–127.
- Reid, K.B.M., 1976. Isolation, by partial pepsin digestion, of the three collagen-like regions present in subcomponent C1q of the first component of human complement. *Biochem. J.* 155, 5–17.
- Reid, K.B.M., 1979. Complete amino acid sequences of the three collagen-like regions present in subcomponent C1q of the first component of human complement. *Biochem. J.* 179, 367–371.
- Reid, K.B.M., 1981. Preparation of human C1q, a subcomponent of the first component of the classical pathway of complement. *Methods Enzymol.* 80, 16–25.
- Sobel, A.T., Bokisch, V.A., 1975. In: Seligman, M., Preud'homme, F.L., Kourilsky, F.M. (Eds.), *Membrane receptors of lymphocytes*. North-Holland, Amsterdam, p. 151.
- Spiro, R.G., Zhu, Q., Bhoyroo, V., Soling, H.D., 1996. Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. *J. Biol. Chem.* 271, 11588–11594.
- Stuart, G.R., Sim, R.B., Malhotra, R., 1996a. Characterisation of radioiodinated lung surfactant protein A (SP-A) and the effects of oxidation on SP-A quaternary structure and activity. *Exp. Lung Res.* 22, 467–487.
- Stuart, G.R., Lynch, N.J., Lu, J., Geick, A., Moffatt, B.E., Sim,

- R., Schwaebler, W.J., 1996b. Localisation of the C1q binding site within C1q receptor/calreticulin. *FEBS Lett.* 397, 245-249.
- Tenner, A.J., 1993. Functional aspects of the C1q receptors. *Behring Inst. Mitt.* 93, 241-253.
- Van den Berg, R.H., Faber-Krol, M., van Es, L.A., Daha, M.R., 1995. Regulation of the function of the first component of complement by human C1q receptor. *Eur. J. Immunol.* 25, 2206-2210.
- White, T.K., Zhu, Q., Tanzer, M.L., 1995. Cell surface calreticulin is a putative mannoside lectin which triggers mouse melanoma cell spreading. *J. Biol. Chem.* 270, 15926-15929.
- Zhu, Q., Zelinka, P., White, T., Tanzer, M.L., 1997. Calreticulin-integrin bidirectional signaling complex. *Biochem. Biophys. Res. Commun.* 232, 354-358.

349175

STIC-ILL

From: Romeo, David
Sent: Monday, June 04, 2001 1:53 PM
To: STIC-ILL
Subject: 09503421 Sharma V K; Agarwal D S; Satyanand; Saha K

3347253

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Sharma V K; Agarwal D S; Satyanand; Saha K
TI Profile of complement components in patients with severe burns.
SO JOURNAL OF TRAUMA, (1980 Nov) 20 (11) 976-8.
Journal code: KAF; 0376373. ISSN: 0022-5282.
AN 81049725

NO 6/4

Profile of Complement Components in Patients with Severe Burns

V. K. SHARMA, M.D., D. S. AGARWAL, M.D., PH.D., SATYANAND, F.R.C.S., AND K. SAHA, PH.D.

Twelve adults hospitalized with extensive burns formed the basis of the present study. On admission, blisterfluid and serum were collected from each patient and subsequently serum only obtained on 3rd, 7th, 14th, 21st, and 28th day of hospital stay. Complement components C1q, C3, C4, and C5 were measured in these samples by single radial immunodiffusion technique. It was observed that immediately after the thermal trauma there was a sudden and profound fall in serum levels of the early and late complement components vis-à-vis their appearance in blisterfluid. However, their levels in the latter were significantly lower than in the former. The mean serum levels of these components gradually increased up to the 14th day after burns and thenceforth leveled off, although normal serum levels for C1q and C3 were never achieved. Three patients died between 6 and 24 days after trauma. Individual variations in the complement profile were observed and no correlation between the serum levels of complement components and prognosis of burned patients was seen.

Severe burns deprive the patient of the mechanical and immunologic protection of his covering integument and mucous membranes. Thermal injury results in impairment of inflammatory reaction (7) and decrease in the phagocytic capacity of neutrophils (6) and reticulo-endothelial system (11). Decrease in serum levels of immunoglobulins following burns has also been reported (1). There is clinical and experimental evidence to suggest suppression of delayed hypersensitivity reactions following thermal trauma (8). In last two decades the structural and functional complexities of the complement system have been defined and its role as a mediator and amplifier of many immune and inflammatory reactions recognized. Complement activity in burned patients has been reported as depressed (2), however, various components of complement have not been analyzed.

MATERIALS AND METHODS

Twelve adults (age range 18 to 35 years, mean age 25.8 years) having extensive burns (more than 30% superficial burns or more than 15% third-degree burns) were chosen for the study. These patients received a uniform regime of treatment. The fluid resuscitation program during the first 24 hours consisted of intravenous administration of sterile normal saline and electrolytes (Sterofundin P, Elys Injekt Ltd., Bombay) calculated on the basis of

percentage of burns and body weight (4 ml/kg/% burn). One half of the calculated volume was given in the first 8 hours after burns and the remaining in next 16 hours. At approximately 24 hours postburn, plasma expanders, namely, dextran injection IP (Lomodex or Dextraven, Rallis-Fison Ltd., West Bengal) in 400 to 800 ml quantity were given. Water orally or as 5% dextrose IV was given in amounts sufficient to maintain urinary output around 50 to 100 ml per hour and blood pressure and hematocrit within normal limits. During the first week of admission one to two units of ABO- and Rh-matched fresh blood were transfused. The burn wounds were wrapped in absorbent dressings containing an inner layer of gauze heavily impregnated with neomycin (Neosporin, Burroughs Wellcome) cream. Burns of head, neck, face, and perineum were treated by exposure after application of a generous layer of neomycin cream. Gentamicin in a cream base was used as topical chemotherapeutic agent against *Pseudomonas* infection. Systemic therapy consisted of crystalline penicillin 5 lac units I.D. given IM during early phase of burns; subsequently sensitivity studies were obtained for any organism recovered from blood culture.

Pulmonary injury associated with burns was not seen in any of the 12 patients. Our patients sustained severe burns due to accident by open fires used in traditional Indian kitchens. None of the patients had any clinical illness before sustaining the thermal injury and were apparently in good health. Three patients died between 6 and 24 days after burns.

On admission, 5 ml of blisterfluid and 5 ml of blood were collected in sterile containers from each patient.

From the Departments of Microbiology and Surgery, Maulana Azad Medical College, and the Department of Microbiology, Govind Ballabh Pant Hospital, New Delhi, India.

Address for reprints: V. K. Sharma, M.D., Block C2A, Pocket 16, House No. 188, Janak Puri, New Delhi, India 110058.

Subsequently, 5 ml of blood were collected from patients on 3rd, 7th, 14th, 21st, and 28th day of stay in hospital. Sera were separated and stored along with blisterfluids at -20°C . The hematocrit value was estimated by Wintrobe's method (4) in all blood samples at the time of collection.

Complement components C1q, C3, C4, and C5 were measured in blisterfluid and serum samples by single radial immunodiffusion technique (12) using monospecific antisera and reference standards. Anti C1q antiserum was obtained from Behring Institute, Frankfurt West Germany, while other antisera along with reference standards were procured from Meloy Laboratories, Springfield, U.S.A. The levels of C1q in the samples were compared with the W.H.O. reference standard serum 67/97 and were expressed in units per 100 ml taking the W.H.O. reference standard serum as 100 units per 100 ml. The levels of C3, C4, and C5 were expressed in mg per 100 ml.

The data were analyzed and statistically evaluated (paired *t*-test of significance at 5% level).

RESULTS

The concentrations of complement components C1q, C3, C4, and C5 in the blisterfluid samples from 12 patients collected on day of admission are shown in Table I.

Table II illustrates the complement profile in serum samples collected from burned patients on admission and subsequently on the 3rd, 7th, 14th, 21st, and 28th day of hospital stay. It was observed that the concentrations of all four components in the blisterfluid were lower than those in the serum samples collected at the same time. This difference was found to be statistically significant (paired *t*-test at 0.05 level). The serum to blisterfluid ratios for C1q, C3, C4 and C5 were 1:0.48; 1:0.69, 1:0.70, and 1:0.61, respectively. The mean serum levels of these four components were lowest in serum samples collected on day of admission. Their levels increased thereafter up to the 14th day and thenceforth leveled off (Table II). When the serum complement profile at admission was compared with that at 28th day, it was observed that the rise in mean levels of complement components C1q, C3,

TABLE I
Mean levels of complement components in the blisterfluid from 12 patients with severe burns

Components	Mean* \pm S.D.† (Range)
C1q	17 \pm 11 (5-35)
C3	64 \pm 39 (10-168)
C4	14.3 \pm 7 (6-27)
C5	6.4 \pm 2.5 (3-12.7)

* The level of C1q was expressed in units per 100 ml; that of C3, C4 and C5 in mg per 100 ml.

† Standard deviation.

TABLE II
Profile of early and late complement components in the sera of 12 patients with severe burns

Serum Sample	Day of collection	Mean* \pm S.D.† (Range)			
		C1q	C3	C4	C5
I	Admission	38 \pm 18 (5-100)	93 \pm 47 (10-190)	20.3 \pm 6.6 (7.5-30)	10.5 \pm 3.3 (4.2-18.5)
II	3rd	44 \pm 16 (25-75)	92 \pm 51 (32-183)	20.6 \pm 6.8 (6-37)	10.6 \pm 4 (5.7-18.2)
III	7th	68 \pm 23 (37-133)	102 \pm 33 (37-142)	23.2 \pm 8.2 (7.5-35)	11.2 \pm 3.4 (6.2-17.2)
IV	14th	74 \pm 19 (30-100)	128 \pm 48 (35-220)	24.2 \pm 7.7 (12.5-35)	12.7 \pm 3.4 (7.5-18.5)
V	21st	73 \pm 21 (30-112)	127 \pm 43 (40-190)	25.6 \pm 7.5 (17.5-43.4)	12.5 \pm 4 (8.2-20.7)
VI	28th	74 \pm 23 (37-125)	129 \pm 48 (50-183)	26.5 \pm 5.5 (18-37.5)	12.5 \pm 2.5 (9.7-18.2)

* The levels of C1q were expressed in units per 100 ml; levels of C3, C4, and C5 in mg per 100 ml.

† Standard deviation.

Normal values of complement components C1q, C3, C4, and C5 in healthy Indian adults from same socioeconomic background as of patients in the present study have been estimated in this laboratory (14) as 137 \pm 48 units per 100 ml, 211 \pm 38 mg per 100 ml, 31 \pm 10 mg per 100 ml, and 11.2 \pm 5.4 mg per 100 ml, respectively.

and C4 in serum samples was statistically significant while it was not so for C5 (paired *t*-test at 0.05 level).

The hematocrit values in serially collected blood samples of five male patients varied from 50.5 \pm 3 on the day of admission to 45.5 \pm 2 on the 28th day of trauma; corresponding values for seven female patients ranged from 45.5 \pm 3.5 to 39 \pm 1. The normal hematocrit value for males is reported (4) as 47 \pm 7 and for females 42 \pm 5. Thus, while burned patients had some hemoconcentration in the vascular compartment soon after trauma, the hematocrit values essentially were within normal limits subsequently.

DISCUSSION

Complement is now known to be comprised of 11 components that make up about 10% of the globulins in normal serum of man. Normal values of complement components C1q, C3, C4, and C5 in healthy Indian adults from the same socioeconomic background as patients in the present study has been estimated in this laboratory (14) as 137 \pm 48 units per 100 ml, 211 \pm 38 mg per 100 ml, 31 \pm 10 mg per 100 ml, and 11.2 \pm 5.4 mg per 100 ml, respectively. Thus it was observed that complement components C1q and C3 in patients with severe burns showed a profound fall in their concentrations (75% for C1q and 65% for C3) and although their levels increased subsequently in 2 weeks by 100% and 70%, their normal levels were not attained even 28 days after trauma (Table II, footnote). The fall in levels of components C4 and C5 was less striking (33% for C4 and 6% for C5) and near-normal levels were achieved in 2 to 3 weeks.

This sudden decrease in concentrations of C1q and C3 in serum within hours after thermal trauma concomitant

with their appearance in blisterfluid indicates increased permeability of the microvasculature (1) resulting in leakage of the high molecular weight protein molecules to burned areas and redistribution of fluid and proteins between edema and intravascular space. Appearance of complement components of molecular weights (10) varying from 200,000 to 400,000 daltons in blisterfluid also suggests severe immediate and sustained damage to the vascular endothelium (5). In our series of cases the clinical conditions were standardized as far as possible; even then therapeutic measures may have precluded to some extent the obtaining of actual data on concentrations of complement components. Infusion of plasma expanders and electrolytes may have contributed to some dilution in their levels and blood transfusions may have had the opposite effect. Decreased levels are unlikely to be associated with hemoconcentration seen in these patients in initial stage of trauma. During the study period due to protein catabolism it is possible that these patients might have developed protein calorie undernutrition influencing the serum complement profile. Although we have not studied the nutritional status of these patients by monitoring serum albumin level, however, the possibility of these patients developing severe nutritional deficit and thus affecting the complement profile could be excluded clinically because all these patients were given a high-protein diet including nutritive infusion of essential amino acids (Hermin injection) to abrogate such deficiency. During the absorption phase of the burns syndrome, lasting 3 to 10 days after burns (1), levels of C1q, C3, C4, and C5 showed a gradual and steady increase in their levels but expected normal levels for C1q and C3 were not attained. Failure to achieve normal concentrations even 4 weeks after sustaining trauma may be due to the interplay of decreased synthesis and increased catabolism as has been observed in earlier studies on serum levels of immunoglobulins following burns (1). A disturbed liver function might also be a contributory factor (9). The decrease in levels of C1q and C3 may also indicate the presence of circulating antigen-antibody complexes in these burned patients (8).

The overall sustained decrease in serum levels of complement components in burns can impede bacteriolysis and dampen amplification of inflammatory reaction. It has been suggested by Artz (3) that the alternate pathway of complement system which is an important defense against *Pseudomonas* infection becomes blocked in patients with extensive burns. During the present study two patients died 6 days after sustaining burns and in both of them the serum concentrations of components C1q and C3 were below 40 units per 100 ml and 50 mg per 100 ml, respectively. A third patient died as a result of *Pseudomonas aeruginosa* septicemia 24 days after admission and had low levels of C1q (30 units per 100 ml) and C3 (50 mg per 100 ml) on the day of admission. These values had risen to 75 units per 100 ml and 125 mg per 100 ml by the 21st day of stay in the burns unit, i.e., before development of fatal infection. On the other hand,

another two patients with initial serum levels of C3 below 50 mg per 100 ml not only survived but also showed an increase in C3 levels up to 180 mg% in 4 weeks of followup. The major surgical maneuvers undertaken during 4 weeks followup were surgical debridement of eschar and application of autografts in four patients. Autografts took well and there was no effect of these procedures on complement profile of these patients. Out of 12 patients in the study two developed bacteremia. *Staphylococcus pyogenes* was responsible for bacteremia in one patient who survived, and *Pseudomonas aeruginosa* was isolated from blood in another patient who died on the 24th day of stay in hospital. Local wound sepsis was also seen in these two patients and four others. One female patient developed a urinary tract infection due to *Escherichia coli*. Analysis of the data showed that the alteration of complement profile during the course of study period had no correlation with bacteremia or local wound sepsis. Thus despite attempts to study a homogenous group of patients, fairly great individual differences in the profile of complement components were observed and it was difficult to have a definite correlation between the prognosis of burned patients and their serum levels of complement components.

Acknowledgment

The authors are grateful to the patients for allowing serial collection of sera and wish to thank (Miss) Rashmi Sharma for her technical assistance.

REFERENCES

1. Arturson, G., Hogman, C. G., Johansson, S. G. O., et al.: Changes in immunoglobulin levels in severely burned patients. *Lancet*, 2: 546-548, 1969.
2. Arturson, G., Fjellstrom, K. E.: Complement-active serum proteins in burns. In *Research in burns, Transactions of the Second International Congress on Research in Burns*, Edinburgh, 1965, p. 401.
3. Artz, C. P.: Burns updated—1975 Fitts Oration. *J. Trauma*, 16: 3-15, 1976.
4. Davidsohn, I., Nelson, D. A.: The Blood. In Davidsohn, I., Hency, J. B. (eds): *Clinical Diagnosis by Laboratory Methods*. Philadelphia, Saunders, 1974, pp. 100-310.
5. Foley, F. D.: Pathology of cutaneous burns. *Surg. Clin. No. Amer.*, 50: 1201-1210, 1970.
6. Grogan, J. B., Miller, R. C.: Impaired function of polymorphonuclear leukocytes in patients with burns and other trauma. *Surg. Gynecol. Obstet.*, 137: 784-788, 1973.
7. Knisely, M. H.: Enforced postponement of selective phagocytosis following burns: A contribution to the biophysics of disease. *Ann. NY Acad. Sci.*, 150: 510-527, 1968.
8. Kohn, J.: Abnormal immune response in burns. *Postgrad. Med. J.*, 48: 335-337, 1972.
9. Kukral, J. C., Shoemaker, W. C.: The metabolic sequelae of burn trauma. *Surg. Clin. No. Amer.*, 50: 1211-1216, 1970.
10. Lachmann, P. J.: Complement. In Gell, P. G. H., Coombs, R. R. A., Lachmann, P. J. (eds): *Clinical Aspects of Immunology*. Oxford Blackwell, 1975, pp. 323-364.
11. Lemperle, G.: Reticuloendothelial system in the burned rat. *J. Infect. Dis.*, 117: 7-14, 1967.
12. Mancini, G., Carbonara, A. C., Heremans, J. P.: Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, 2: 235-259, 1965.
13. Munster, A. M.: Alterations of the host defense mechanism in burns. *Surg. Clin. No. Amer.*, 50: 1217-1234, 1970.
14. Saha, K., Chakraborty, A. K.: Serum complement profile in human leprosy and its comparison with immune complex diseases. *Int. J. Lep.*, 45: 327-337, 1977.

STIC-ILL

From: Romeo, David
Sent: Monday, June 04, 2001 1:33 PM
To: STIC-ILL
Subject: 09503421 Pinckard R N; Olson M S; Giclas P C; Terry R; Boyer J T; O'Rourke R A

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Pinckard R N; Olson M S; Giclas P C; Terry R; Boyer J T; O'Rourke R A
TI Consumption of classical complement components by heart subcellular
membranes in vitro and in patients after acute myocardial
infarction.
SO JOURNAL OF CLINICAL INVESTIGATION, (1975 Sep) 56 (3) 740-50.
Journal code: HS7; 7802877. ISSN: 0021-9738.
AN 76006071

Consumption of Classical Complement Components by Heart Subcellular Membranes In Vitro and in Patients after Acute Myocardial Infarction

R. NEAL PINCKARD, MERLE S. OLSON, PATRICIA C. GICLAS, RICHARD TERRY,
JOHN T. BOYER, and ROBERT A. O'ROURKE

*From the Departments of Microbiology, Biochemistry, and Medicine,
University of Arizona College of Medicine, Tucson, Arizona 85724, and the
Department of Medicine, College of Medicine, University of California,
San Diego, California 92103*

ABSTRACT Experiments were conducted to characterize the antibody-independent activation of complement in human serum by isolated human heart mitochondrial membranes in vitro and to determine whether similar patterns of complement consumption occurred in patients after acute myocardial infarction. Direct evidence for the interaction of C1 and heart mitochondrial membranes was obtained by mitochondria-C1 binding and elution experiments. Exposure of normal human sera to isolated human heart mitochondria at 37°C resulted in the consumption of C1, C4, C2, and C3 without significant consumption of the terminal components of the complement system (C6 through C9). The consumption occurred in the absence of detectable anti-heart mitochondria autoantibody, was demonstrated to be calcium dependent, and was inhibited by either 0.01 M EDTA or ethylene glycol bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA). Although specific absorption of C1q from human sera inhibited the mitochondria-dependent activation of C4, C3 consumption was not affected. These data indicate that the consumption of C4 and C2 likely occurred due to the mitochondrial membrane-mediated activation of C1, but that the consumption of the C3 did not necessarily involve either the classical or alternative complement pathways. After the in vitro characterization of the mitochondria-dependent activation of the complement system, additional studies were performed to determine whether similar consumption occurred in patients after acute myocardial infarction. During a 72-h period after

hospital admission significant decreases in C1, C4, and C3 occurred in six patients after acute myocardial infarction but not in six patients with recent chest pain but no evidence of acute myocardial infarction. These studies suggest that myocardial cell necrosis results in the release of subcellular membrane constituents capable of activating the complement system in the absence of detectable anti-heart autoantibodies; such activation may be responsible in part for the development of acute inflammation and evolution of the infarct size following coronary artery occlusion.

INTRODUCTION

The processes involved in the destruction of myocardial tissue after coronary artery occlusion have been the subject of intensive investigation. Myocardial cell necrosis following coronary artery occlusion is thought to include an initial autolytic and a subsequent heterolytic destruction of myocardial cells in the area of the infarction (1). The autolytic destruction of tissue likely is due to a restricted perfusion of the affected area resulting in a decreased delivery of oxygen and substrates necessary for normal myocardial energy generation. After the initial autolytic tissue damage of heterolytic destruction of myocardial tissue occurs associated with inflammatory cell infiltration into the affected area. During the first 48 h after myocardial infarction the neutrophil is the predominant inflammatory cell within the evolving myocardial lesion and likely is involved in the heterolytic phase of myocardial damage.

There are a variety of factors which could mediate the neutrophilic infiltration following tissue damage,

Received for publication 31 December 1974 and in revised form 13 May 1975.

one of which is the complement system of serum proteins. Hill and Ward (2) have demonstrated that the neutrophilic infiltration following experimental myocardial infarction in rats was reduced significantly by the prior administration of cobra venom factor, which causes a depletion of the third component (C3) of serum complement. In view of the above, it is crucial to elucidate mechanisms governing the activation of complement after myocardial cell damage.

There are presently three known mechanisms for the activation of the complement system: (a) the classical activation of complement due to the activation of C1 by IgG or IgM antibody; (b) the activation of the alternative pathway by a variety of substances capable of interacting with properdin; and (c) the direct proteolytic activation of C1 and C3 by enzymes such as plasmin or trypsin.

We initiated studies to determine whether anti-heart autoantibodies might mediate the classic activation of complement after acute myocardial infarction. It was shown that IgM, complement-fixing, anti-heart mitochondria autoantibody developed 7-14 days after experimental myocardial infarction in dogs (3, 4); similar temporal production of anti-heart mitochondria autoantibodies was observed in patients after acute myocardial infarction.¹ However, the time-course of autoantibody development precluded its involvement in the initial inflammatory response during the first 2-3 days after myocardial infarction. Subsequently, we demonstrated that all human serum from subjects without prior history of myocardial disease contained a heat-labile, heart-reactive factor which, after the interaction with isolated human heart mitochondrial membranes, led to the activation of serum complement (5). This serum factor was identified as the first component of serum complement which bound directly to the mitochondrial membrane and resulted in an antibody-independent activation of the complement system.

The present studies were designed to characterize further the mechanism(s) for the antibody-independent activation of complement by the heart subcellular membranes and to determine whether a similar intravascular activation of complement occurs in patients after acute myocardial infarction.

METHODS

Preparation of heart mitochondrial membranes. Mitochondrial membranes were prepared from human heart tissue obtained at autopsy within 1 h of the time of death. Portions of the left ventricle were sectioned and washed with a solution containing 0.25 M sucrose and 0.01 M Tris-chloride, pH 7.4, at 0-4°C. This buffer was pre-equilibrated with 100% oxygen before the cardiac tissue was washed. Washed tissue was passed through a meat

grinder (2-mm holes) into approximately 7 vol of the same sucrose-Tris buffer. The resultant mince was disrupted further with a Polytron PT 20 tissue homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.) at setting 5 for 45 s. The homogenate was centrifuged at 600 g for 10 min. The sediment was discarded and the supernate was recentrifuged at 5,500 g for 15 min. Each mitochondrial pellet was washed twice with the sucrose-Tris buffer (20-25 ml per pellet) and finally resuspended in the same buffer to a final protein concentration of approximately 10 mg/ml. The mitochondrial suspension was frozen rapidly in 0.5-ml aliquots and stored at -70°C. Before freezing, the functional integrity of each mitochondrial preparation was determined by measuring the respiratory control ratio with pyruvate and malate as substrates and ADP as the respiratory stimulant (6). Respiratory control ratios of between 5.0 and 7.5 were normal for these human heart mitochondria preparations.

Measurement of anti-heart mitochondria autoantibody. Anti-heart mitochondria autoantibody was assessed by a micro-complement fixation test using a microtiter apparatus (Cooke Engineering Co., Alexandria, Va.). Doubling dilutions of heat-inactivated (56°C for 30 min) human sera were made in 25 μ l of 0.15 M sodium barbital-buffered saline, pH 7.3, containing 0.1% human albumin. Complement fixation was determined by the addition of 25 μ l of a dilution of guinea pig serum containing 1.50 minimal hemolytic doses of complement followed by 25 μ l of isolated human heart mitochondria antigen (1 mg mitochondrial protein/ml). The reaction was allowed to proceed at 37°C for 30 min and then 25 μ l of a 1.5% suspension of sensitized sheep erythrocytes was added and the reaction was allowed to proceed at 37°C for 40 min. The microtiter plates were centrifuged at 1,000 rpm for 5 min and the degree of hemolysis was estimated by visual inspection. The autoantibody titer was defined as the highest dilution of serum at which incomplete hemolysis had occurred. A serum was considered negative for anti-heart mitochondria autoantibody if the titer was less than 1:2. The optimal concentration of the heart mitochondria antigen was determined by checkerboard titrations using a standard serum containing anti-heart mitochondria autoantibody. Appropriate serum and antigen controls were performed simultaneously with the above assays. A positive anti-heart mitochondria autoantibody serum was titrated along with every test in order to ensure uniformity in the sensitivity of the complement fixation test from day to day.

Preparation of complement fixation reagents. Sensitized sheep red blood cells (EA),² Veronal-buffered saline ($\Gamma/2 = 0.15$) containing 0.1% gelatin (GVB), and low ionic strength GVB ($\Gamma/2 = 0.065$) containing 3% glucose (Gl-GVB) were prepared according to the methods outlined by Rapp and Borsos (7). Partially purified human C1 was prepared by euglobulin fractionation of fresh human serum as described previously (5). Sera congenitally deficient in the 4th, 6th, or 7th components of complement were ob-

² Abbreviations used in this paper: BSA, bovine serum albumin; CH₅₀, total hemolytic complement; CPK, creatinine phosphokinase; EA, sensitized sheep red blood cells; ECG, electrocardiogram; EGTA, ethylene glycol bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid; Gl-GVB, low ionic strength GVB ($\Gamma/2 = 0.065$) containing 3% glucose; GOT, glutamic oxaloacetic transaminase; GVB, Veronal-buffered saline ($\Gamma/2 = 0.15$) containing 0.1% gelatin; KCNS, potassium thiocyanate; LDH, lactic dehydrogenase; PBS, phosphate-buffered saline.

¹ Pinckard, R. N., M. S. Olson, and R. A. O'Rourke. Unpublished observations.

tained from C4-deficient guinea pigs, C6-deficient rabbits, and a C7-deficient human, respectively.

Human-serum deficient in C3, C4, and C5 but containing stable, oxidized C2 was prepared by treating serum with potassium thiocyanate (KCNS) followed by iodination.³ 1 vol of 2.0 M KCNS was added to 1 vol of fresh human serum and the mixture was allowed to incubate overnight at 0°C. The mixture was dialyzed for 2 h against distilled water and for an additional 2 h each against three changes of phosphate-buffered saline, pH 6.8 (PBS). 9 vol of the KCNS-treated serum were reacted with 1 vol of triatomic iodine (I₂) for 5 min at 0°C. The reaction mixture was dialyzed overnight against PBS and subsequently against GVB. The dialyzed oxy-KCNS-treated serum was aliquoted and frozen in an acetone-dry ice bath and stored at -70°C. The I₂ was prepared by the addition of 0.3173 g of I₂ (Allied Chemical Corp., Morristown, N. J.) to 25.75 g of KI and the resulting solution brought to a final volume of 100 ml with distilled water. Human serum deficient in C3 and C4 was prepared by the treatment of 1 vol of fresh serum with 1 vol of 1.0 M KCNS as described above.

Preparation of cellular intermediates. EAC4 cells were prepared according to the method of Borsos and Rapp (8). EAC1 cells were formed by incubation at 0°C for 15 min of 200-400 effective molecules of human or guinea pig C1 with EA (5×10^5 cells/ml). To this mixture was added 2 vol of the human serum diluted 1:10 with GVB containing 0.01 M EDTA. Alternatively human serum heated to 56°C for 30 min was used in place of the human serum-0.01 M EDTA-GVB mixture. The EAC1.4 cells were washed three times each with 0.01 M EDTA-GVB and with GVB and finally washed two times with GI-GVB.

EAC1.4oxy-2 cells were prepared as described previously.³ EAC1 cells were prepared by the addition of sufficient C1 to 20 ml of 2.5% suspension of EA to yield approximately 400 effective molecules of C1 per erythrocyte. After a 20-min incubation at 37°C, the EAC1 cells were washed 2 times in warm (37°C) GVB and resuspended to the original volume. 10 ml of heat-inactivated (56°C for 30 min) human serum was added and the suspension was incubated 30 min at 37°C. The EAC1.4 cells were washed 3 times and were resuspended to the original volume. 10 ml of oxy-KCNS-treated serum was added to the EAC1.4 cells followed by incubation for 5 min at 37°C. The EAC1.4oxy-2 cells were washed 2 times in GI-GVB and adjusted to a concentration of 1×10^8 cells/ml in GI-GVB.

Complement assays. Total hemolytic complement (CH₅₀), C1, C2, C8, and C9 titrations were performed according to procedures described by Cordis Laboratories (Miami, Fla.) utilizing cellular intermediates and functionally purified human complement components. C4, C6, and C7 titrations were performed by utilizing C4-deficient guinea pig serum, C6-deficient rabbit serum, and C7-deficient human serum, respectively. The C4, C6, and C7 assays were performed by the addition of 0.1 ml of serum dilutions to 0.1 ml GI-GVB followed by the addition of 0.1 ml of optimal dilutions of the appropriate serum deficient in the respective complement component (C4, 1:50; C6, 1:12.5; C7, 1:30). 0.1 ml of sensitized erythrocytes (1×10^8 EA/ml) was added and the suspension was incubated for 120 min at 37°C with intermittent shaking. C3 assays were performed by the addition of 0.1 ml of serum dilutions (prepared in GI-GVB) to 0.1 ml of GI-GVB and 0.1 ml of EAC1.4oxy-2 cells (1.0×10^8

cells/ml). The mixture was incubated at 30°C for 30 min with intermittent shaking. 0.1 ml of a dilution of serum depleted of C3 and C4 but containing excess C5-C9 (references 9, 10; 0.50 M KCNS-treated serum) then was added and the mixture incubated at 37°C for 60 min. At the end of the incubation period for each of the above assays 1 ml of cold saline was added, and after centrifugation the supernates were analyzed spectrophotometrically at 415 nm to quantify the percent hemoglobin released. The 50% hemolytic end-point was determined by the von Krogh equation (7) and the results expressed as the number of functional hemolytic complement component units per milliliter of undiluted serum.

Patient selection. The clinical studies were performed on patients admitted to the Coronary Care Unit and Myocardial Infarction Research Unit at the University Hospital, University of California School of Medicine, San Diego. After patient admission, a peripheral venous line (19-gauge angiocatheter) was inserted into a vein in the wrist or arm for subsequent withdrawal of blood samples and for the intravenous injection of medication when needed. The line was kept open with heparin (1,000 U/20 ml flush solution). Blood samples for creatine phosphokinase (CPK), glutamic oxaloacetic transaminase (GOT), lactic dehydrogenase (LDH), and complement determinations were obtained on admission, hourly for the first 7 h, at 2-h intervals for the remainder of the first 24 h, and subsequently at 4-h intervals until 72 h had elapsed. Subsequent blood samples were obtained once daily. All blood samples were allowed to clot at room temperature and the serum aliquots were stored at -70°C for subsequent determination of complement levels and the presence of anti-heart mitochondria autoantibodies. A diagnosis of acute myocardial infarction was based upon the three following criteria: (a) a history of prolonged chest pain typical of acute myocardial infarction; (b) evolutionary electrocardiographic (ECG) changes indicative of acute transmural myocardial infarction; and (c) a rise and subsequent fall in serum levels of CPK, GOT, and LDH in the characteristic pattern of acute myocardial infarction. Six of the patients included in the present studies with evidence of acute myocardial infarction met all three of these criteria. Six other patients were admitted to the Coronary Care Unit for observation because of recent chest pain; none of these patients had evolutionary ECG changes or subsequent elevations in serum enzyme levels. Two of the control patients had angina pectoris without evidence of infarction, two had atypical chest pain with chest wall tenderness, one had a hiatal hernia with reflux esophagitis, and there was no apparent etiology for chest pain in the sixth patient. The four patients without angina subsequently had no ECG changes during progressive exercise treadmill testing and none of the six patients had pleuritis or pericarditis.

RESULTS

C1 binding to heart mitochondria. Direct evidence for the binding to C1 to the mitochondrial membrane was obtained by C1 binding and elution experiments (Table I). Partially purified human C1 was adjusted to an ionic strength of 0.15 followed by incubation at 37°C for 15 min in order to activate C1. Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml of the activated C1 preparation at 0°C. After a 15-min incubation the mitochondria were

³ Boyer, J. T., and P. Wyde. 1975. Hypochlorite-induced alterations of human serum complement. Submitted for publication.

TABLE I
C1 Binding to Heart Mitochondria

	Hemolytic C1	
	U/ml	
Purified C1	115,000	
C1 remaining in the supernate	32,000	
C1 eluted from mitochondria	120,000	

Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml of a partially purified preparation of activated C1 at 0°C and were incubated for 15 min. The mitochondria were removed by centrifugation and were washed two times with GI-GVB, $r/2 = 0.065$. After the second wash the mitochondria were resuspended in 0.5 ml of 0.30 M NaCl and were incubated at 0°C for 15 min. The mitochondria were removed by centrifugation and the supernate was diluted with an equal volume of distilled water. Hemolytic C1 assays were performed on the original unabsorbed C1 preparation, the supernate from the mitochondria-absorbed C1 preparation, and the C1 eluted from the mitochondrial membrane.

removed by centrifugation (17,000 *g* for 2 min) and were washed 2 times with GI-GVB, $r/2 = 0.065$. After the second wash bound C1 was dissociated from the mitochondria by resuspension in 0.5 ml of 0.30 M NaCl and incubation at 0°C for 15 min. The mitochondria were removed by centrifugation and the supernate was diluted with an equal volume of distilled water in order to establish an ionic strength of $r/2 = 0.15$. Hemolytic C1 assays were performed on the original unabsorbed C1 preparation, the supernate from the mitochondria-absorbed C1 preparation, and the C1 eluted from the mitochondrial membrane. In numerous experiments, one of which is shown in Table I, absorption of C1 preparations with isolated human heart mitochondria always removed greater than 70% of the hemolytic C1 activity; significant C1 activity was recovered from the mitochondria-C1 complex after incubation in 0.30 M NaCl. Similar experiments conducted with fresh human serum also indicated the ability of 4 mg isolated human heart mitochondria to absorb greater than 50% of the C1 from 1 ml undiluted human serum.

Mitochondrial membrane-induced consumption of individual serum components. Studies were conducted to determine which components of complement were consumed after exposure of sera to isolated human heart mitochondria. Isolated human heart mitochondria, 4 mg mitochondrial protein, were resuspended in 0.5 ml of fresh undiluted normal human sera. These sera were documented not to contain detectable complement-fixing, anti-heart mitochondria autoantibody as described in Methods. The mixture was incubated at 37°C for 30 min and the mitochondria were removed by

centrifugation at 17,000 *g* for 2 min. Sera not exposed to isolated human heart mitochondria, but incubated at 37°C for 30 min served as controls. A typical experiment is shown in Table II and demonstrates that significant consumption of C1, C4, C2, and C3 occurred in serum exposed to isolated human heart mitochondria. In contrast, significant consumption of the terminal components of complement (C6 through C9) was never observed. The CH₅₀ decreased to a similar degree as the decreases in the hemolytic levels of C2 and C3. When similar experiments were performed in the presence of 0.01 M EDTA, no consumption of C4, C2, or C3 was observed although a significant decrease in C1 was observed.

Fig. 1 demonstrates the rate of consumption of C4 in serum exposed to isolated human heart mitochondria. Fresh human serum was exposed to isolated human heart mitochondria (8 mg mitochondrial protein/ml undiluted serum) at 37°C. At various times samples were removed and centrifuged at 17,000 *g* for 2 min and the C4 titers were determined on the supernates. Sera not exposed to human heart mitochondria but treated in a similar manner served as controls. It can be seen that, immediately after exposure of serum to isolated human heart mitochondria, there was a precipitous decrease in C4 level. The consumption of C4 continued until only 10% of the original C4 level was present 30 min after exposure of the serum to isolated

TABLE II
Consumption of Complement in Human Serum 30 min after the Addition of Isolated Human Heart Mitochondria

Complement component	Serum control	Serum-mitochondria	Percent reduction
hemolytic U/ml serum			
C1	152,000	60,000	61
C4	1,900,000	185,000	90
C2	7,000	5,600	20
C3	320,000	220,000	31
C6	1,300	1,200	8
C7	60,000	53,000	12
C8	1,480,000	1,530,000	0
C9	440,000	400,000	9
CH ₅₀	1,250	910	27

Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml of fresh undiluted normal human serum documented not to contain detectable complement-fixing, anti-heart mitochondria autoantibody. The mixture was incubated at 37°C for 30 min and the mitochondria were removed by centrifugation. Serum not exposed to isolated mitochondria but incubated at 37°C for 30 min served as control. Hemolytic assays were performed on the serum control and serum exposed to mitochondria and the percent reduction of the individual complement components was determined.

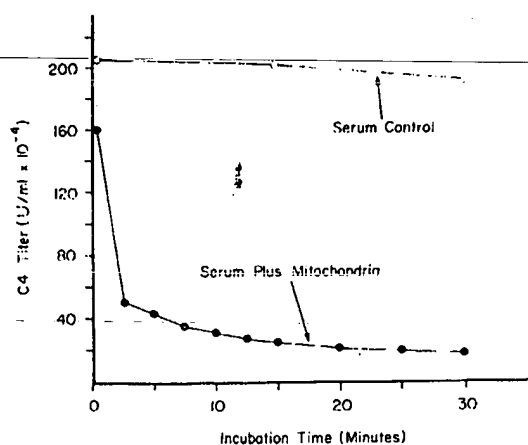


FIGURE 1 Rate of C4 consumption in fresh human serum exposed to isolated human heart mitochondria. Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 1.0 ml of fresh human serum documented not to contain any detectable anti-heart mitochondria autoantibody. The suspension was incubated at 37°C and at various times samples were removed and centrifuged and C4 levels were determined on the supernates. Serum not exposed to human heart mitochondria was treated in a similar manner and served as a control.

human heart mitochondria. As in the previous experiment the presence of 0.01 M EDTA prevented the mitochondria-dependent consumption of C4 in human serum.

Additional experiments were performed to determine whether the mitochondria-dependent consumption of C3

TABLE III
Effect of EGTA upon the Heart Mitochondria-Dependent Consumption of C4 and C3 in Normal Human Sera

Experiment	Complement component	
	C4	C3
	U/ml	
Without EGTA		
Serum control	900,000	200,000
Serum-mitochondria	140,000 (84%)*	125,000 (37%)
Serum-zymosan	650,000 (28%)	55,000 (72%)
With 0.01 M EGTA		
Serum control	710,000	190,000
Serum-mitochondria	720,000 (0%)	175,000 (8%)
Serum-zymosan	800,000 (0%)	92,000 (56%)

Isolated human heart mitochondria (4 mg mitochondrial protein) were resuspended in 0.5 ml fresh human serum in the presence or absence of 0.01 M EGTA at 37°C and were incubated for 30 min. The mitochondria were removed by centrifugation and the hemolytic levels of C4 and C3 were determined.

* Percent reduction when compared to control serum.

occurred as a result of classical activation of complement or was due to activation of the alternative complement pathway. In a representative experiment (Table III) fresh human serum, 0.5 ml, was exposed to 4 mg of human heart mitochondria in the presence or absence of 0.01 M EGTA at 37°C for 30 min. The mitochondria were removed by centrifugation (17,000 *g* for 2 min) and the hemolytic levels of C4 and C3 were determined. Serum which was not exposed to isolated human heart mitochondria or which was exposed to 4 mg of zymosan served as controls, the latter being performed to demonstrate the activation of the alternative pathway under the experimental conditions. As can be seen in Table III, significant consumption of C4 and C3 occurred in serum not containing EGTA that was reacted with either isolated human heart mitochondria or zymosan. In contrast, no significant consumption of either C4 or C3 occurred in serum containing EGTA that was reacted with mitochondria, but significant consumption of C3 occurred in the serum containing EGTA and zymosan.

Although the above experiments indicated repeatedly that mitochondria-dependent consumption of C3 did not occur through activation of the alternative pathway, they did not establish that the consumption of C4 or C3 by mitochondria occurred through the activation of the classic complement pathway. Therefore, additional experiments were performed to determine whether or not the mitochondria-dependent consumption of C4 and C3 required the activation of human C1 by human heart mitochondria. In a representative experiment (Table IV), fresh human serum was absorbed with rabbit anti-bovine serum albumin (BSA)-BSA immune complexes in slight antibody excess in the presence of 0.01

TABLE IV
Mitochondria-Dependent Consumption of C4 and C3 in Normal and C1q-Absorbed Human Sera

Experiment	Complement component	
	C4	C3
	U/ml	
Normal		
Serum control	970,000	290,000
Serum-mitochondria	130,000 (87%)*	190,000 (34%)
C1q-absorbed		
Serum control	900,000	170,000
Serum-mitochondria	750,000 (17%)	120,000 (30%)

Isolated human heart mitochondria (4 mg mitochondria protein) were resuspended in 0.5 ml of fresh human serum or human serum depleted of C1q. After incubation at 37°C for 30 min the mitochondria were removed by centrifugation and the C4 and C3 levels were determined.

* Percent reduction when compared to control serum.

f comple-
e-comple-
it (Table
to 4 mg
or absence
ochondria
r 2 min)
re deter-
ed human
4 mg of
performed
e pathway
e seen in
id C3 oc-
t was re-
ochondria
nsumption
ng EGTA
icant con-
containing

repeatedly
C3 did not
pathway,
of C4 or
tivation of
additional
whether or
of C4 and
man heart
nt (Table
with rabbit
nune com-
ice of 0.01

id C3 in

ponent

C3

0,000
0,000 (34%)

0,000
0,000 (30%)

mitochondria
ian serum or
at 37°C for
fugation and
rum.

M EDTA in order to remove C1q selectively. Three absorptions with BSA-anti-BSA-immune complexes consistently removed greater than 95% of the functional C1 activity. 4 mg isolated human heart mitochondria was resuspended in 0.5 ml of the recalcified C1q-depleted human serum. After incubation at 37°C for 30 min, the mitochondria were removed by centrifugation (17,000 g for 2 min) and the hemolytic levels of C4 and C3 were determined. Fresh human sera not absorbed with BSA-antiBSA immune complexes were utilized in similar experiments and served as controls. As seen in Table IV, removal of C1q significantly decreased the mitochondria-dependent consumption of C4 but did not reduce significantly the consumption of C3.

Intravascular consumption of complement components in patients after acute myocardial infarction. Studies were performed to determine whether similar consumption of complement components also might occur in patients after acute myocardial infarction. Serial serum samples were obtained from patients during the 72-h period following hospitalization. 12 patients were included in these studies: 6 patients with unequivocal evidence for acute transmural myocardial infarction; and 6 patients with recent chest pain but who subsequently were shown not to have had an acute myocardial infarction. CH₅₀, C1, C4, C3, and C6 assays were performed on the sera from each patient simultaneously. The maximum percent decreases, during the initial 72-h period following hospital admission, in each respective complement component were determined relative to the levels of the individual complement components determined for the serum sample obtained at admission. The data in Table V show the maximum percent decreases in CH₅₀, C1, C4, C3, and C6 and the times at which these decreases occurred in six control patients

TABLE V
Maximum Percent Decrease in Complement during the 72-h Period Following Hospitalization of Control Patients without Acute Myocardial Infarction

Patient	Complement component				
	CH ₅₀	C1	C4	C3	C6
T. O.	20* (7)	12 (5)	17 (7)	0 (0)	11 (9)
E. E.	17 (4)	11 (4)	14 (36)	15 (8)	10 (36)
K. A.	12 (20)	11 (12)	4 (4)	17 (35)	4 (8)
H. B.	24 (12)	7 (14)	19 (14)	11 (6)	12 (6)
R. A.	0 (0)	25 (4)	ND	14 (10)	5 (2)
R. G.	1 (40)	7 (4)	10 (36)	26 (10)	0 (0)

* Maximum percent decrease observed relative to complement levels of the serum obtained at admission; values shown in parentheses indicate the time (hours) after hospitalization at which the maximum percent decrease in complement occurred. ND, assay not done.

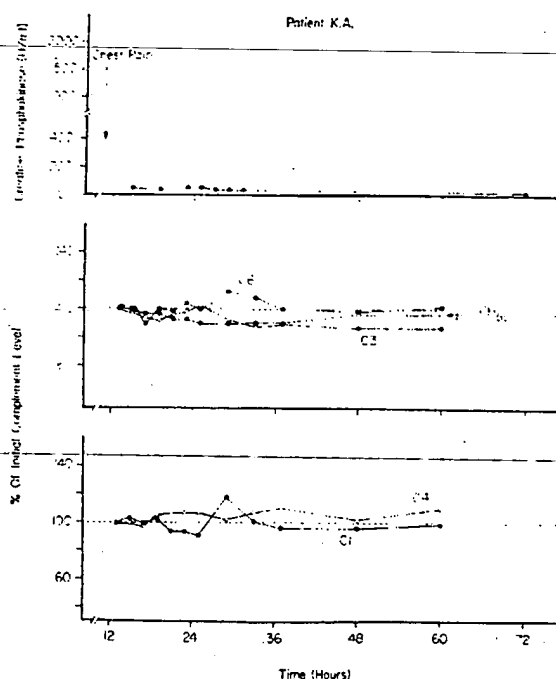


FIGURE 2 Temporal changes in the percent decreases in the levels of C1, C4, C3, C6, and CH₅₀ in relation to the serum CPK in control patient K. A., who did not experience acute myocardial infarction. The time scale in this figure is constructed so that 12 h represents noon on the day of admission and 72 h represents midnight of the 3rd day.

with chest pain but without myocardial infarction. None of the six patients had detectable anti-heart mitochondria antibody in any of the sera studied. These six patients were chosen as controls since the environmental influences and methods for the procurement of blood samples and serum preparation were identical to the six patients with acute myocardial infarction. The data in Table V demonstrate that there were no large decreases in any of the components measured in any of the six patients studied. The mean decreases for the CH₅₀ and C1 were 12% (range 0-24% and 7-25%); the mean decreases of C4 and C3 were 13 and 14%, respectively (range 4-19% and 0-28%); and for C6 it was 7% (range 0-12%). In addition there was no relationship either between patients or within individual patients of the times at which the maximum decreases in individual complement components occurred.

Fig. 2 depicts the fluctuations in the serum levels of CH₅₀, C1, C4, C3, and C6 in a representative control patient. Patient K. A. was admitted 2 h after the onset of atypical chest pain and had no evidence of acute myocardial infarction; i.e. there were neither elevations in the serum levels of CPK, GOT, or LDH, nor evolu-

TABLE VI
Maximum-Percent-Decrease-in Complement during the 72-h
Period Following Hospitalization of Patients
with Acute Myocardial Infarction

Patient	Complement component				
	CH ₅₀	C1	C4	C3	C6
E. H.	ND	65* (41)	38 (41)	33 (24)	0 (0)
C. G.	17 (22)	53 (22)	62 (22)	26 (22)	13 (22)
J. C.	33 (28)	50 (21)	18 (21)	28 (21)	0 (0)
L. C.	13 (18)	27 (18)	32 (18)	29 (22)	15 (4)
A. W.	5 (21)	47 (21)	44 (8)	47 (21)	10 (2)
J. E.	25 (6)	45 (26)	29 (26)	47 (10)	6 (0)

* Maximum percent decrease observed relative to complement levels of the serum obtained at admission; values shown in parenthesis indicate the time (hours) after hospitalization at which the maximum percent decrease in complement occurred. ND, assay not done.

tionary ECG evidence of acute myocardial infarction. No significant fluctuations in the levels of complement components measured were observed over a 50-h period following admission.

In striking contrast to the control patients, significant decreases in the levels of C1, C4, and C3 occurred in every patient with acute myocardial infarction during the 72-h period following hospitalization. These six patients were admitted to the hospital 2-4 h after the onset of chest pain. The initial elevations in serum CPK occurred 3-7 h after admission. None of the six patients with acute myocardial infarction had detectable anti-heart mitochondria antibody at the time of admission nor during the following 72 h of hospitalization; however, four of these six patients developed detectable anti-heart mitochondria antibody during the 2nd wk after hospitalization. The data in Table VI demonstrate that there were significant decreases in complement components C1, C4, and C3. The mean decreases observed were as follows: CH₅₀, 18.5% (range 5-33%); C1, 47.8% (range 27-65%); C4, 37.2% (range 18-62%); C3, 35% (range 26-47%); and C6, 7.3% (range 0-15%). In contrast to the control patients, there appeared to be a relationship between and especially within patients of the times at which the maximum decreases of C1, C4, and C3 occurred. As can be seen in Table VI the maximum decreases of these components occurred approximately 24 h after hospitalization; furthermore, in five of six patients the maximum decreases in C1 and C4 occurred simultaneously and the maximum decrease in C3 generally occurred within hours of the C1 and C4 decreases. Although the maximum decreases in CH₅₀ tended to correlate temporally with the C1, C4, and C3 decreases, no correla-

tion was observed with respect to the relatively small maximum decrease in C6.

Fig. 3 demonstrates the precipitous decreases in complement components C1, C4, and C3 observed in patient A. W., who had an acute myocardial infarction. The initial decreases in C1, C4, and C3 occurred 43 h after the onset of chest pain during a period of time when the initial increase in serum CPK levels was occurring. The maximum decreases in C1, C4, and C3 occurred within 24 h and returned to within normal limits during the 3rd day of hospitalization. The CH₅₀ and C6 levels did not fluctuate significantly during the first 48 h after admission, but tended to become elevated on the 3rd day after hospitalization.

Table VII shows a statistical comparison of the maximum percent decreases during the 72-h period following hospitalization in the serum levels of CH₅₀, C1, C4, C3, and C6 between the control and experimental groups. The data, obtained from Tables V and VI, were analyzed by the Student's *t* test for the comparison of means between two groups. These comparisons documented significant decreases in the levels of C1, C4, and C3 ($P < 0.001$, < 0.001 , and < 0.005 , respectively)

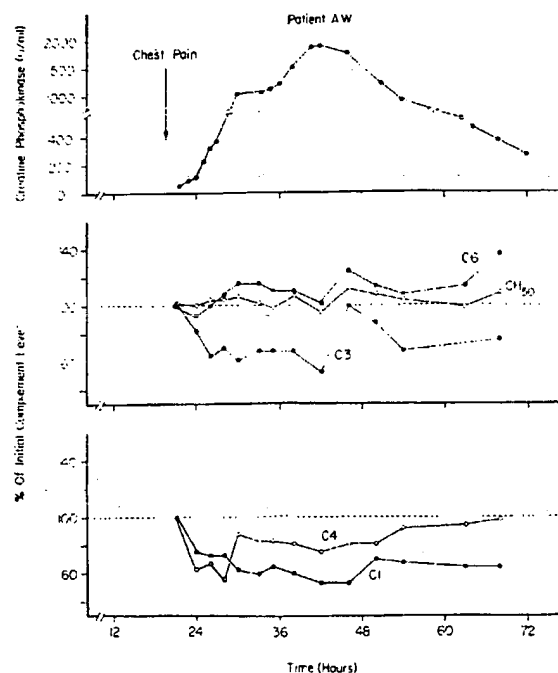


FIGURE 3 Temporal changes in the percent decreases in the levels of C1, C4, C3, C6, and CH₅₀ in relation to the elevation of the serum CPK in patient A. W. This patient was documented to have experienced unequivocal acute transmural myocardial infarction. The time scale in this figure is constructed so that 12 h represents noon on the day of admission and 72 h represents midnight of the 3rd day.

TABLE VII
Statistical Comparison (Student's *t*) of Maximum Complement Decreases during the 72-h Period Following Hospitalization in Patients with and without Acute Myocardial Infarction

	Mean \pm SD of percent maximum decrease of complement components				
	CH ₅₀	C1	C4	C3	C6
Patients with acute myocardial infarction	18.6 \pm 10.9 (<i>P</i> > 0.3)*	47.8 \pm 12.4 (<i>P</i> < 0.001)	37.2 \pm 15.0 (<i>P</i> < 0.001)	35.0 \pm 9.6 (<i>P</i> < 0.005)	7.3 \pm 6.4 (<i>P</i> > 0.9)
Control patients without myocardial infarction	12.3 \pm 10.0	12.2 \pm 6.6	12.8 \pm 6.0	13.8 \pm 8.5	7.0 \pm 4.7

* *P* value determined by the Student's *t* test for the comparison of means between two groups; the data utilized in these analyses are given in Tables V and VI.

between the patients with acute myocardial infarction and the control patients without myocardial infarction. No significant differences were noted between the two groups with respect to the maximum percent decreases in CH₅₀ or levels of C6.

DISCUSSION

Recent evidence indicates that myocardial cell necrosis following coronary artery occlusion can be reduced significantly by a variety of therapeutic interventions. Agents which decrease myocardial oxygen demand, increase the supply of oxygen to the myocardium, enhance anaerobic myocardial metabolism, or augment the transport of oxygen and substrates to ischemic cells seems to prevent or to attenuate the autolytic and heterolytic destruction of normal myocardial tissue located in the marginal zone of the infarct (1, 11). Although in-patient deaths from acute myocardial infarction due to primary arrhythmias have been reduced significantly, reduction in the morbidity and mortality due to the destruction of functional myocardial tissue following coronary artery occlusion until recently has not been possible. This latter point is of special importance in view of the correlation between infarct size and prognosis (12). Therefore, it is apparent that the molecular factors governing the evolution of infarct size after coronary artery occlusion warrant further investigation so that appropriate therapy may be developed to limit the degree of myocardial cell necrosis.

The manipulation of factors that affect the development of acute inflammation may prove to be relevant to the above considerations. Interventions have been designed to prevent the autolytic destruction of myocardial cells and the heterolytic destruction of normal myocardial tissue following the infiltration of inflammatory cells. For example, it has been demonstrated that corticosteroid administration can lead to the reduction of infarct size in the experimental animal (13, 14). Presumably the action of corticosteroids is mediated through their ability to stabilize membranes preventing autolysis and by their anti-inflammatory properties

which prevent the heterolytic destruction of normal myocardial tissue caused by infiltrating inflammatory cells, particularly the neutrophilic polymorphonuclear leukocyte. Another promising means of affecting the evolution of infarct size by suitable modification of acute inflammation derives from the ability of cobra venom factor to reduce the amount of myocardial damage after experimental myocardial infarction in dogs (15). Cobra venom factor activates the alternative complement pathway, resulting in significant depletion of C3, which would be expected to prevent a wide variety of inflammatory reactions especially involving the neutrophil. In addition, C3 depletion also would prevent the generation directly or indirectly of a variety of vasoactive substances.

The importance of complement in the development of acute myocardial inflammation following coronary artery occlusion has been studied in the rat. These studies have demonstrated that the infiltration of neutrophils after experimental myocardial infarction is dependent upon C3 (2). These studies also demonstrated that the C3-dependent neutrophil infiltration likely was initiated by the release of a C3 converting enzyme from damaged myocardial tissue and that such inflammation could be prevented experimentally by the administration of cobra venom factor. The C3-converting enzyme was shown to cleave directly either purified rat or human C3 resulting in the generation of C3 leukotactic factors. C3-converting activity was demonstrated in tissue minces of rat, heart, lung, and spleen (16) and in saline extracts of extensively homogenized rat heart tissue (2).

In view of the possibility that factors released from damaged myocardial tissue may be involved in the activation of the complement system and subsequent development of the acute inflammatory process, our laboratories have been studying a possible immunologic involvement in the pathogenesis of myocardial damage in a variety of cardiac diseases. Our initial interests centered around the temporal development of anti-heart autoantibody after experimentally induced myo-

cardial infarction in dogs (3). However, it was evident that due to the temporal development of the anti-heart mitochondria autoantibody, autoantibody was not involved in the initial inflammatory process occurring within the first 24-72-h period following coronary artery occlusion. Subsequently, we demonstrated a 19S complement-fixing, heat-labile factor present in all normal human serum, which was reactive with human heart mitochondria and sarcoplasmic reticulum. This serum factor reacted with autologous and homologous heart mitochondria but did not react with mitochondria derived from cardiac tissue of other species. Subsequent characterization indicated that this heat-labile factor was the first component of human complement. The C1 reacted with the mitochondrial membrane, was activated, and resulted in an apparent antibody-independent activation of the complement system (5).

The present studies were undertaken to characterize further the mitochondria-dependent activation of complement in human serum and to determine whether similar activation of serum complement also occurred in patients after acute myocardial infarction. Evidence was obtained for the direct binding of partially purified human C1 to the mitochondrial membrane by C1 fixation and elution experiments (Table I). It is likely that the binding of C1 to the mitochondrial membrane occurred through the C1q component since previous studies have demonstrated the ability of isolated mitochondria to selectively remove C1q from human sera in the presence of 0.01 M EDTA (5). These observations, together with the experiments which demonstrated that selective absorption of C1q from human serum prevented the mitochondria-dependent consumption of C4 (Table IV), indicated that direct C1 binding occurred on the mitochondrial membrane with subsequent activation of the C1 molecule.

Incubation of isolated human heart mitochondria with human serum at 37°C resulted in a consumption of C1, C4, C2, and C3 without significant consumption of the terminal components of the complement system, C6 through C9 (Table II). The mitochondria-dependent consumption of C4 was calcium dependent and was inhibited by the presence of 0.01 M EDTA or EGTA, (Table III). These data, together with the significant inhibition of C4 consumption in C1q-depleted serum (Table IV), indicated that the C4 consumption was dependent upon activation of C1 by mitochondria. The inhibition by EGTA of the mitochondria-dependent consumption of C3 indicated that the C3 consumption was not due to activation of the alternative complement pathway by heart mitochondria; under the same experimental conditions significant C3 consumption occurred after exposure of serum to zymosan (Table III). However, no decrease in C3 consumption occurred

in C1q-depleted serum, which suggests that the consumption of C3 was independent of C1 activation (Table IV). These experiments do not completely exclude the possible role of C1 since the C1q-depleted serum contained small amounts of functional C1 and some mitochondria-dependent consumption of C4 was noted. It is unlikely that the decreases in C3 in the serum exposed to mitochondria were due simply to C3 absorption on the mitochondrial membrane for two reasons. First, either EDTA or EGTA inhibited the mitochondria-dependent consumption of C3 in the serum. Second, in contrast to serum not exposed to mitochondria, serum exposed to mitochondria consistently revealed the conversion of $\beta 1c$ to $\beta 1a$. Therefore, it is possible that the mitochondria-dependent consumption of C3 may be analogous to the C3 consumption documented by Hill and Ward (2), who demonstrated a C3-converting enzyme derived from myocardial tissue that enzymatically fragmented human C3. Proteolytic consumption of C3 might explain the lack of C6-C9 consumption as it has been documented that the C3-dependent activation of C5 requires the formation of a C423 complex or C3 associated with factor B of the alternative pathway (17).

After the characterization of the activation of the first four components of human complement by isolated human heart mitochondria, studies were performed to determine whether similar complement decreases occurred in patients after acute myocardial infarction. Sequential serum samples for six patients with unequivocal myocardial infarction were assessed for significant fluctuations in the functional levels of C1, C4, C3, C6, and CH₅₀ during the 72-h period following hospitalization. The data demonstrated significant decreases in the functional levels of C1, C4, and C3 but not C6 or CH₅₀ in all six patients. The initial precipitous decreases in the functional levels of C1, C4, and C3 occurred simultaneously with the initial elevation in serum CPK (Fig. 3). In general, the maximum decreases in the functional levels of these complement components occurred within 24 h after hospitalization. These maximum decreases in C1 and C4 occurred simultaneously in five of six patients with the maximum decrease in C3 occurring within hours of the maximum decreases in C1 and C4 (Table VI). In contrast, no significant decreases in functional levels of the complement components studied occurred in six control patients with chest pain but without myocardial infarction. The small fluctuations in complement components in the control patients did not change precipitously nor was there any apparent diurnal variation (Table V, Fig. 2). Statistical comparison of the maximum percent decreases in the functional levels of various complement components in the myocardial infarction

t the con-
 sion (Table
 exclude the
 serum con-
 some mito-
 noted. It is
 an exposed
 sorption on
 ions. First,
 tochondria-
 Second, in
 dria, serum
 ed the con-
 ble that the
 3 may be
 ted by Hill
 urning-en-
 zymatically
 tion of C3
 n as it has
 ctivation of
 plex or C3
 ve pathway

tion of the
 ent by iso-
 were per-
 complement de-
 ocardial in-
 atients with
 ssessed for
 vels of C1,
 d following
 nificant de-
 and C3 but
 tial precipi-
 C1, C4, and
 al elevation
 aximum de-
 complement
 spitalization.
 occurred si-
 ie maximum
 ie maximum
 contrast, no
 of the com-
 six control
 rdial infarc-
 ent compo-
 nge precipi-
 al variation
 of the maxi-
 vels of vari-
 rdial infarc-

tion and control groups during the first 72 h of hos-
 pitalization showed a high degree of significance with
 respect to C1, C4, and C3 but not with respect to CH₅₀
 or C6 (Table VII).

The present studies are the first documentation of
 significant decreases in the functional levels of in-
 dividual complement components in patients immedi-
 ately after acute myocardial infarction. Alterations in
 total serum complement have been reported previously
 in patients after myocardial infarction (18). However,
 in these studies it was reported that patients with myo-
 cardial infarction had abnormally high levels of CH₅₀
 2-5 days after myocardial infarction. Recently it has
 been reported that hypercatabolism of various comple-
 ment components in a variety of diseases is accom-
 panied by a hypersynthesis of these same complement
 components (19). In view of the results of the present
 study it is possible that the elevated CH₅₀ levels previ-
 ously reported (18) were due to an initial consumption
 of complement immediately after myocardial infarction
 with the subsequent compensatory hypersynthesis of
 these complement components.

We have demonstrated that isolated human heart
 mitochondria can activate the first four components of
 human complement in human serum *in vitro* and that
 similar decreases in the functional levels of these serum
 complement components occur in patients after acute
 myocardial infarction. Although these data indicate the
 intravascular activation of the first four components of
 complement after acute myocardial infarction, unequivocal
 documentation for the intravascular consumption of
 plasma complement can only be obtained by measuring
 the fractional catabolic rates of these components (19)
 and/or documenting the development of circulating
 complement conversion products. The decreases of in-
 dividual complement components both *in vitro* and *in vivo*
 in the present studies could not be explained on
 the basis of the presence of anti-heart mitochondria
 autoantibody. Whether the release of subcellular organ-
 elles from damaged myocardial tissue is responsible for
 the antibody-independent decrease in the functional
 levels of individual complement components in patients
 after myocardial infarction is not known at present.
 Certainly a wide variety of substances have been re-
 ported to activate C1 or C3 in the absence of detectable
 antibody and include single- and double-stranded DNA,
 various polynucleotides, proteolytic enzymes such as
 trypsin and plasmin, endotoxin, certain viruses, poly-
 cations, and C-reactive protein (17). Regarding C-re-
 active protein, it is noteworthy that the initial and max-
 imum decreases in C1, C4, and C3 reported in the pres-
 ent studies occurred before the reported increase in
 C-reactive protein and other acute phase reactants in
 myocardial infarction patients (20).

In view of the recent evidence indicating the role
 of complement in the development of acute myocardial
 inflammation and evolution of infarction size (1, 2,
 15), the molecular mechanisms governing the activation
 of complement by heart subcellular organelles war-
 rants further investigation. Such studies might pro-
 vide new information regarding potential therapeutic
 interventions which could selectively inhibit the activa-
 tion of complement after heart damage, thereby de-
 creasing the amount of myocardial cell necrosis fol-
 lowing coronary artery occlusion.

ACKNOWLEDGMENTS

We thank Ms. Janice Guttery for her excellent technical
 assistance.

This work was supported by National Institutes of
 Health grants HL 14467 and HL 18194 and the Arizona
 Heart Association.

REFERENCES

1. Braunwald, E., and P. R. Maroko. 1974. The reduction
 of infarct size—an idea whose time (for testing) has
 come. *Circulation*. 50: 206-209.
2. Hill, J. H., and P. A. Ward. 1971. The phlogistic role
 of C3 leukotactic fragments in myocardial infarcts of
 rats. *J. Exp. Med.* 133: 885-900.
3. Pinckard, R. N., M. S. Olson, R. A. O'Rourke, J. D.
 Palmer, R. E. Kelley, and S. Goldfein. 1971. Develop-
 ment of complement-fixing 19S, anti-heart mitochondria
 autoantibody following myocardial infarction in dogs.
Circ. Res. 29: 276-285.
4. Kelley, R. E., M. S. Olson, and R. N. Pinckard. 1974.
 Characterization of an anti-heart mitochondria autoanti-
 bodies produced in dogs following myocardial infarction.
Circ. Res. 35: 862-870.
5. Pinckard, R. N., M. S. Olson, and R. E. Kelley, D. H.
 DeHeer, J. D. Palmer, R. A. O'Rourke, and S. Gold-
 fein. 1973. Antibody-independent activation of human
 C1 after interaction with heart subcellular membranes.
J. Immunol. 110: 1376-1382.
6. Chance, B., and G. R. Williams. 1956. The respiratory
 chain and oxidative phosphorylation. *Adv. Enzymol.*
 17: 65-134.
7. Rapp, H. J., and J. Borsos. 1970. Molecular Basis of
 Complement Action. Appleton-Century-Crofts, New
 York. 164 pp.
8. Borsos, T., and H. J. Rapp. 1967. Immune hemolysis:
 a simplified method for the preparation of EAC'4 with
 guinea pig or with human complement. *J. Immunol.*
 99: 263-268.
9. Dalmaso, A. P., and H. J. Müller-Eberhard. 1966.
 Hemolytic activity of lipoprotein-depleted serum and
 the effect of certain anions on complement. *J. Immunol.*
 97: 680-685.
10. Cooper, N. R., M. J. Polley, and H. J. Müller-Eber-
 hard. 1971. Biology of complement. In *Immunological
 Disease*. M. Samter, editor. Little, Brown and Com-
 pany, Boston. 2nd edition. 289-331.
11. Maroko, P. R., and E. Braunwald. 1973. Modification
 of myocardial infarction size after coronary occlusion.
Ann. Intern. Med. 79: 720-733.
12. Sobel, B. E., G. F. Bresnahan, W. E. Shell, and R. D.
 Yoder. 1972. Estimation of infarct size in man and its
 relation to prognosis. *Circulation*. 46: 640-648.

13. Spath, J. A., Jr., D. L. Lane, and A. M. Lefer. 1974. Preservation of cardiac cellular integrity by methylprednisolone in acute myocardial ischemia. *Am. J. Cardiol.* 33: 171. (Abstr.)
14. Libby, P., P. R. Maroko, C. M. Bloor, B. E. Sobel, and E. Braunwald. 1973. Reduction of experimental myocardial infarct size by corticosteroid administration. *J. Clin. Invest.* 52: 599-607.
15. Maroko, P. R., and C. B. Carpenter. 1974. Reduction in infarct size following acute coronary occlusion by the administration of cobra venom factor. *Clin. Res.* 22: 289. (Abstr.)
16. Hill, J. H., and P. A. Ward. 1969. C3 leukotactic factors produced by a tissue protease. *J. Exp. Med.* 130: 505-518.
17. Cooper, N. R. 1973. Activation of the complement system. In *Contemporary Topics in Molecular Immunology*. R. A. Reisfeld and W. J. Mandy, editors. Plenum Publishing Corporation, New York. 2: 155-183.
18. Boltax, A. J., and F. E. Fischel. 1956. Serologic tests for inflammation. Serum complement, C-reactive protein and erythrocyte sedimentation rate in myocardial infarction. *Am. J. Med.* 20: 418-427.
19. Ruddy, S., C. B. Carpenter, K. W. Chin, J. N. Knostman, N. A. Soter, O. Götze, H. J. Müller-Eberhard, and K. F. Austen. 1975. Human complement metabolism: an analysis of 144 studies. *Medicine (Baltimore)*. 54: 165-178.
20. Johansson, B. G., C. O. Kindmark, E. Y. Trelle, and F. A. Wollheim. 1972. Sequential changes of plasma proteins after myocardial infarction. *Scand. J. Clin. Lab. Invest. Suppl. 121*. 29: 117-126.

349179

STIC-ILL

From: Romeo, David
Sent: Monday, June 04, 2001 1:26 PM
To: STIC-ILL
Subject: 09503421 Ghebrehiwet B; Galanakis D K

3347224

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Ghebrehiwet B; Galanakis D K
TI Clq inhibitor (chondroitin-4-sulfate proteoglycan):
structure and function.
SO BEHRING INSTITUTE MITTEILUNGEN, (1993 Dec) (93) 214-23. Ref: 18
Journal code: 9KI; 0367532. ISSN: 0301-0457.
AN 94226571

10/4

C1q Inhibitor (Chondroitin-4-Sulfate Proteoglycan): Structure and Function

B. GHEBREHIWET^a§, D. K. GALANAKIS§

The Departments of ^aMedicine and §Pathology, State University of New York, N. Y. 11794-8161, USA

Summary

The serum C1q inhibitor (C1q INH) is a chondroitin 4-sulfate proteoglycan which is composed of several polyanionic components ranging in size from 21-750 kDa. Although the activity of C1q INH has been described in terms of its ability to precipitate C1q and inhibit its hemolytic activity, not much is known about either the mechanisms of its action or its role in health and disease. This report provides evidence that a 30 kDa core protein component of the proteoglycan macromolecule contains most of the C1q inhibitory activity. This inhibitory activity occurs as a result of C1q INH binding to the C1q "heads" (gC1q) as well as to the collagen "tail" (cC1q). What may be more significant in terms of perpetuation of inflammatory processes is the ability of C1q INH to moderately activate the classical pathway leading to C2 and C4 consumption. The binding of C1q INH to C1q is enhanced at low ionic strength, but significant binding does occur under physiologic conditions which makes it likely for the inhibitor to participate in inflammatory processes especially in microenvironments of high inhibitor concentration. Such elevated concentration does occur in patients with active rheumatoid arthritis and systemic lupus erythematosus either as a result of unregulated proteoglycan synthesis or disturbances in connective tissue metabolism. Another important function of serum C1q INH is its ability to prolong the clotting time of plasma and fibrinogen solutions containing or lacking CaCl₂. This potent anticoagulant activity is again displayed by the 30 kDa putative protein core which specifically binds to both the E and D domains of fibrinogen. However, the epitope(s) on the 30 kDa which binds to C1q appears to be distinct from that which binds to fibrinogen. The known presence of proteoglycans on the basement membranes and other sites may explain at least in part the presence of fibrinogen in atheromatous lesions. Furthermore, by binding to fibrinogen, soluble C1q INH—and C1q-C1q INH complexes may limit fibrin gelation in inflammatory and tissue repair microenvironments.

Key-Words: C1q inhibitor, proteoglycan, chondroitin 4-sulfate.

Introduction

The existence of a molecule which inhibits the hemolytic activity of C1q was first reported by Conradie et al.¹ These authors demonstrated that a polyanionic macromolecule was associated with C1q when the purification method of Yonemasu and Stroud² was employed. This macromolecule could be dissociated from C1q by affinity chromatography using Concanavalin A (Con A) covalently linked to Sepharose 4B. Because the binding site of Con A is specific for α -D-mannosyl, α -D-glucosyl and steri-

cally similar residues and C1q contains both type of sugars² the application of purified C1q² to Con A-Sepharose 4B results in the binding of C1q while the polyanionic macromolecule comes through unadsorbed¹. Such purified C1q was found to have an enhanced hemolytic activity, and, conversely, addition of the polyanionic macromolecule to C1q inhibited its hemolytic activity¹ hence the designation of C1q inhibitor (C1q INH)¹. Furthermore, this macromolecule has the unique

property of precipitating by Ouchterlony analysis that of antigen-antibody inhibitory properties of other demonstrated when C1q-mediated cellular abrogated if the C1q-co-cyte target cells were first fixed C1q-INH before ex-cells⁵.

The serum C1q INH was tri et. al.⁴ to be a chondro-glycan and probably origi-nal tissues, vessel walls a report is intended to no-the low hemolytic activity-arations may be due to INH but also to highligh-ic relevance of this mole-interaction with C1 and

Purification of C1q Inh

The purification of C1q-mally present in normal-effected by several steps-precipitations in the pre-lowed by affinity chrom-Sephadex 4B essentially-minor modifications¹. B-ed blood is allowed to clo-for 1 hr., after which, the-and placed at 4 °C for an-a retraction to occur and-centrifugation (1400 × g-natant (serum) is remove-an additional 40 min at 4-the serum is added 1/4-0.1M EDTA pH 7.5 and-37 °C). Next, the mixture-freezer until the serum te-down to 0 °C, and the pH-0.1N NaOH or 0.1N H-EDTA pH 7.5 is added-the ionic strength of-4 (I=0.04). The mixture-ice, for 1 hr, is stirred e

: Structure and Func-

11794-8161, USA

glycan which is composed of although the activity of C1q inhibit its hemolytic activity, le in health and disease. This proteoglycan macromolecule occurs as a result of C1q "tail" (cC1q). What may be is the ability of C1q INH to mption. The binding of C1q ing does occur under physio- in inflammatory processes, elevated concentration does pus erythematosus either as nnective tissue metabolism. ig the clotting time of plasma ticoagulant activity is again binds to both the E and D b binds to C1q appears to be roteoglycans on the basement f fibrinogen in atheromatous d C1q-C1q INH complexes vironments.

ues and C1q contains both the application of purified epharose 4B results in the hile the polyanionic macro- through unadsorbed¹. Such found to have an enhanced, and, conversely, addition of acromolecule to C1q inhib- activity¹ hence the designa- tor (C1q INH)¹. Further- omolecule has the unique

property of precipitating C1q when examined by Ouchterlony-analysis in a manner similar to that of antigen-antibody interaction^{3,4}. The inhibitory properties of C1q-INH were further demonstrated when it was shown that C1q-mediated cellular cytotoxicity could be abrogated if the C1q-coated chicken erythrocyte target cells were first precoated with purified C1q-INH before exposure to the effector cells⁵.

The serum C1q INH was identified by Silvestri et. al.⁴ to be a chondroitin 4-sulfate proteoglycan and probably originates from peripheral tissues, vessel walls and blood cells⁶. This report is intended to not only emphasize that the low hemolytic activity of certain C1q preparations may be due to the presence of C1q INH but also to highlight the pathophysiological relevance of this molecule by discussing its interaction with C1 and fibrinogen.

Purification of C1q Inhibitor

The purification of C1q INH, which is normally present in normal human serum, can be effected by several steps of low ionic strength precipitations in the presence of EDTA² followed by affinity chromatography on Con A-Sepharose 4B essentially as described but with minor modifications¹. Briefly, freshly collected blood is allowed to clot at room temperature for 1 hr., after which, the clotted blood is sliced and placed at 4 °C for an additional 1 hr for clot retraction to occur and the clot removed by centrifugation (1400 × g, 30 min). The supernatant (serum) is removed and centrifuged for an additional 40 min at 4 °C and 16,000 × g. To the serum is added 1/4 the serum volume of 0.1M EDTA pH 7.5 and incubated (15 min at 37 °C). Next, the mixture is placed at -70 °C freezer until the serum temperature has cooled down to 0 °C, and the pH adjusted to 7.5 using 0.1N NaOH or 0.1N HCl and then 0.005M EDTA pH 7.5 is added while stirring until the ionic strength of the mixture reaches 4 (I=0.04). The mixture, which is kept on ice, for 1 hr, is stirred every 20 min for ap-

proximately 2 min each after which it is placed in clear plastic bottles and centrifuged at 16,000 × g for 30 min at 4 °C. The serum is siphoned off and the precipitate which is visible on the sides of the flasks is washed with 2X the original volume of serum using 0.1M EDTA, pH 7.5 (I=0.04) by centrifuging at 16,000 × g for 30 min at 4 °C. The resulting precipitate is dissolved in 12 ml (per 100 ml of original serum volume) of 0.7M NaCl-0.01M EDTA pH 5.0 and left at 4 °C overnight. The solution is then centrifuged (30,000 × g, 30 min, 4 °C) to remove aggregates and the supernatant dialyzed against 1000 ml 0.1M EDTA pH 5.0 (= 0.078) for 2 hr with one change of buffer and dialysis overnight at 4 °C. The precipitate is collected by centrifugation (16,000 × g, 30 min, 4 °C), washed twice in the same EDTA buffer using 1/2 the original volume of serum and the precipitate dissolved in approximately 5 ml of 0.3M NaCl-0.005M EDTA pH 7.5. This solution is then centrifuged at 30,000 × g (30 min, 4 °C) and the supernatant contains "relatively" pure C1q as assessed by SDS-PAGE and Coomassie staining. This is because, the C1q-INH which remains associated with C1q up to this stage stains very poorly with Coomassie and therefore gives the impression that the C1q is devoid of other molecules. The C1q-INH is separated from C1q by passage over a Con A-Sepharose 4B column which is equilibrated with 10mM Veronal buffer pH 8.0 containing 0.5M NaCl, 1mM CaCl₂, 1mM MgCl₂ and 0.02% NaN₃. The C1q-INH passes unadsorbed to the column whereas the C1q which is bound to the column, is eluted with 10% methyl-α-D-mannopyranoside (Sigma) in equilibration buffer (Fig. 1).

Assay for C1q-INH

Fractions containing C1q INH can be identified by either single⁷ or double⁸ radial diffusion in agarose^{3,4} using a 1% agarose matrix in isotonic phosphate-buffered saline pH 7.4 containing 1.5% polyethylene glycol. After 24 hr at room temp., a precipitin line develops

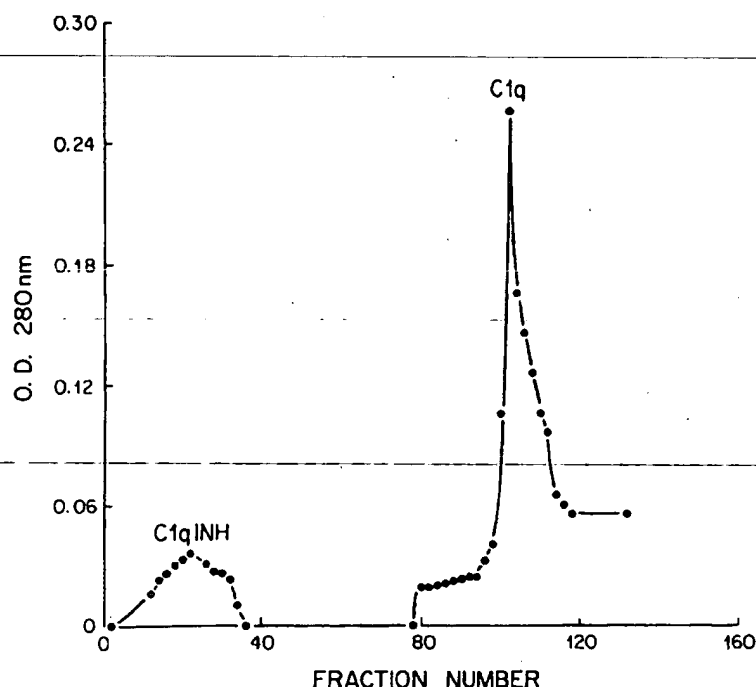


Fig. 1: Dissociation of C1q INH from C1q. The final C1q preparation obtained by low ionic precipitations in the presence of EDTA⁷ was applied to Con A-Sepharose 4B. The C1q INH passes through the column and adsorbed whereas the bound C1q is eluted using a 10% solution of methyl- α -D-mannopyranoside.

when C1q interacts with C1q-INH. Alternatively, C1q-INH containing fractions can be identified by a hemolytic assay using C1q-depleted or deficient serum (C1q-D) as described⁹. Briefly, the sample containing C1q-INH is mixed with GVB (isotonic Veronal-buffered saline containing 1.5mM CaCl₂ and 5mM MgCl₂ and 0.1% gelatin) and 5–10 ng C1q in a final volume of 300 μ l GVB and then incubated for 30 min at 37°C. Then, 10 μ l C1qD serum and 200 μ l EA (1.5×10^8 /ml) are added and the samples incubated for an additional 60 min at 37°C. After incubation, the reaction mixture is stopped by addition of 1 ml cold GVB, centrifuged and hemoglobin released into the supernatant measured spectrophotometrically at 412 nm. The C1q-INH activity is calculated as the percent inhibition of C1q hemolytic activity^{3,9} and is illustrated in Table 1. If complete C1q-deficient serum is not avail-

able, C1q-depleted serum can be made by passage of normal human serum containing 10mM EDTA over an IgG-Sepharose CL4B and the unbound material collected, concentrated to original volume, and after reconstitution with Ca²⁺ and Mg²⁺ by addition of 20 μ l/ml C1qD from a stock solution composed of 0.3M CaCl₂ and 1M MgCl₂, used as C1qD reagent⁹.

Structural and Biochemical Features of C1q INH

The C1q INH is a polydisperse, polyanionic macromolecule which was identified by *Silvestri et al.*⁷ to be a chondroitin 4-sulfate proteoglycan and like many proteoglycans, does not stain with either Coomassie blue or Amido black. However, Alcian blue, which has specificity for glycosaminoglycans, has been

Table 1 Inhibition of C1q hemolysis

C1q INH added (μ l/10 ng C1q)
2*
5
7
10
20

* 10 μ l of C1q INH was approximately incubated with increasing amounts of C1q (30 min, 37°C) followed by 10 μ l of C1q (1.5 $\times 10^8$ /ml). After incubation, the supernatant was measured at 412 nm. The C1q activity was calculated as the percent inhibition of C1q hemolysis. The results are the mean of three runs in duplicates.

used to stain chondroitin sulfate proteoglycans at an electrolyte concentration that minimizes staining of other glycoproteins^{10,11}. Typical Alcian blue containing 0.2% Alcian blue in 0.1M sodium chloride, 0.025 M acetic acid, v/v ethanol/water is used. When nitrocellulose paper is electrotransferred proteoglycans are stained. The strips are soaked (30 min at room temperature) (3 \times 15 min) in the above Alcian blue¹¹.

A typical preparation of C1q INH contains about 22% uronic acid, 12% sulfate and 9% radiolabeled C1q INH by autoradiography reveals the macromolecule consisting of high molecular weight species ranging from 5–15% gradient gels) to resemble cartilage proteoglycans. C1q INH does not form a complex with chondroitin 6-sulfate and its glycosaminoglycan content is void of chondroitin 6-sulfate suggesting that the source of the

Table 1 Inhibition of C1q hemolytic activity

C1q INH added (μ l/10 ng C1q)	Inhibition (%)
2*	5
5	9
7	21
10	47
20	82

* 10 μ l of C1q INH was approximately 5 μ g. C1q (10 ng) was incubated with increasing amounts of C1q INH (30 min, 37 °C) followed by additional incubation (60 min, 37 °C) with 10 μ l of C1q D serum and 200 μ l EA (1.5×10^8 /ml). After incubation, hemoglobin released into the supernatant was measured spectrophotometrically at 412 nm. The C1q INH activity is calculated as the percent inhibition of C1q hemolytic activity. The results are the mean of two separate experiments run in duplicates.

used to stain chondroitin 4-sulfate proteoglycans at an electrolyte concentration which minimizes staining of other negatively charged glycoproteins^{10,11}. Typically, a staining solution containing 0.2% Alcian blue in 0.05M magnesium chloride, 0.025 M sodium acetate, 50% v/v ethanol/water is employed¹¹ especially when nitrocellulose paper strips containing electrotransferred proteoglycans are to be stained. The strips are soaked in staining solution (30 min at room temperature) and destained (3×15 min) in the above buffer but without Alcian blue¹¹.

A typical preparation of serum C1q INH contains about 22% uronic acid, 20% hexosamine, 12% sulfate and 9% protein⁴. Analysis of radiolabeled C1q INH by SDS-PAGE and autoradiography reveals that it is a polydisperse macromolecule consisting of several molecular weight species ranging from 750 kDa (seen on 5–15% gradient gels) to 21 kDa (Fig. 2). Unlike cartilage proteoglycans, the serum C1q INH does not form a complex with hyaluronic acid and its glycosaminoglycan chains are devoid of chondroitin 6-sulfate isomers suggesting that the source of the serum proteoglycan

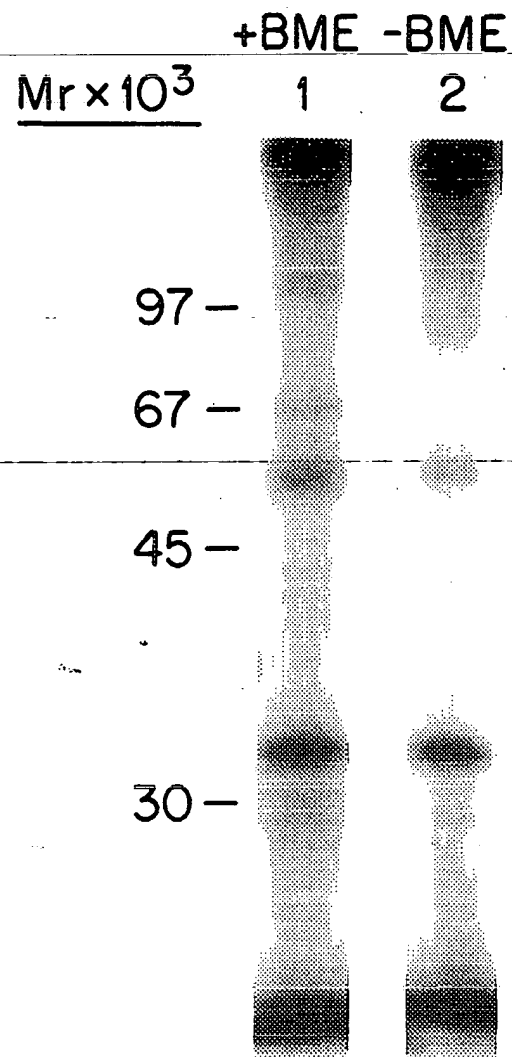


Fig. 2: SDS-PAGE analysis of ¹²⁵I-C1q INH. The C1q INH was radiolabeled by the method of Bolton and Hunter (as described in ref. 3) and analyzed by 10% SDS-PAGE and autoradiography.

is non-cartilaginous in nature⁴. The source of C1q INH has not been identified, however, the fact that platelets and leukocytes produce fully sulfated chondroitin 4-sulfate makes it plausible to assume that part of the C1q INH in plasma is derived from these cells¹². In fact, it was assumed initially, that the plasma C1q INH was a membrane C1q receptor which

160

low ionic precipitations in the pres-
he column anadsorbed whereas the

d serum can be made by pas-
human serum containing
er an IgG-Sepharose CL4B
material collected, concen-
volume, and after reconstitu-
i Mg²⁺ by addition of 20 μ l/
stock solution composed of
M MgCl₂, used as C1qD re-

Biochemical Features of

a polydisperse, polyanionic
hich was identified by Sil-
a chondroitin 4-sulfate pro-
: many proteoglycans, does
ier Coomassie blue or Ami-
er, Alcian blue, which has
cosaminoglycans, has been

was shed off as the cells aged or released as new receptors were synthesized^{3,13}. Subsequent purification of the membrane C1q receptor revealed that proteoglycan was indeed associated or coeluted with the receptor as assessed by the presence of uronic acid and galactosamine but lacked sensitivity to digestion with chondroitinase ABC suggesting that it was not a chondroitin sulfate¹⁴.

Functions Associated with C1q INH

(a) Effect on the complement system

The C1q INH is capable not only of inhibiting the hemolytic activity of C1q but to a smaller degree, even of initiating C1 activation (unpublished data). This indicated that the C1q INH might bind not only to the collagen region of C1q which is physiologically occupied by C1r₂ C1s₂ tetramer but also to the globular

"heads" of C1q as well. Solid phase binding experiments performed with ¹²⁵I-C1q INH and microtiter well immobilized ligands-C1q, C1q "tail" (cC1q), and C1q "heads" (gC1q)-showed that the inhibitor is able to bind strongly to C1q, and gC1q and only moderately to cC1q. Although the binding was optimal at subphysiologic ionic strengths ($I < 0.15$), significant binding takes place even at physiologic ionic strength (Fig. 3).

In order to identify the molecular weight species through which C1q INH bound to C1q or its major fragments, solid phase binding assay was performed as above, and the bound radioactivity eluted by boiling for 5 min in SDS-PAGE sample buffer and then analyzed by SDS-PAGE and autoradiography. As shown in Figure 4, one prominent band with a molecular weight of approximately 30 kDa and two

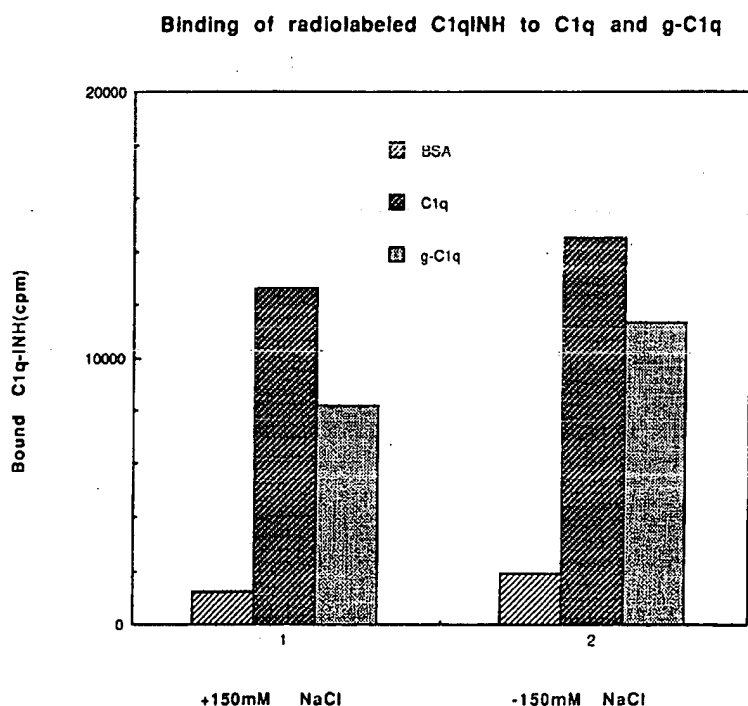


Fig. 3: Solid phase binding assay. Microtiter wells were coated with 50 μ l of 50 μ g/ml C1q or 50 μ g/ml gC1q by incubating for 60 min at 37°C. After incubation, the wells were washed with TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20), ¹²⁵I C1q INH added and further incubated for 60 min at 37°C. The wells are then washed and bound radioactivity determined.

$Mr \times 10^3$

97.5 -

67 -

45 -

30 -

2.1 -

Fig. 4: SDS-PAGE analysis of the bound radioactivity from Figure 3, and the bound radioactivity analyzed by SDS-PAGE (10% gels) bound to C1q and gC1q respectively.

minor bands with molecular weights of approximately 30 kDa and 21 kDa were noted with previous work which showed that the activity could be isolated on a gel filtration column. The activity and analysis by SDS-PAGE and autoradiography revealed the presence of a single chain with a molecular weight of approximately 30 kDa, which probably represents the C1q protein although no ex-

well. Solid phase binding extended with ^{125}I -C1q-INH and mobilized ligands-C1q, C1q C1q "heads" (gC1q)-show or is able to bind strongly to id only moderately to cC1q. ling was optimal at subphynghths ($I < 0.15$), significant ce even at physiologic ionic

y the molecular weight spe-
i C1q INH bound to C1q or
is, solid phase binding assay
above, and the bound radio-
boiling for 5 min in SDS-
ffer and then analyzed by
itoradiography. As shown in
ninent band with a molecu-
roximately 30 kDa and two

1q or 50 $\mu\text{g/ml}$ gC1q by incubating
1Cl, pH 7.5, 150 mM NaCl, 0.05%
are then washed and bound radio-

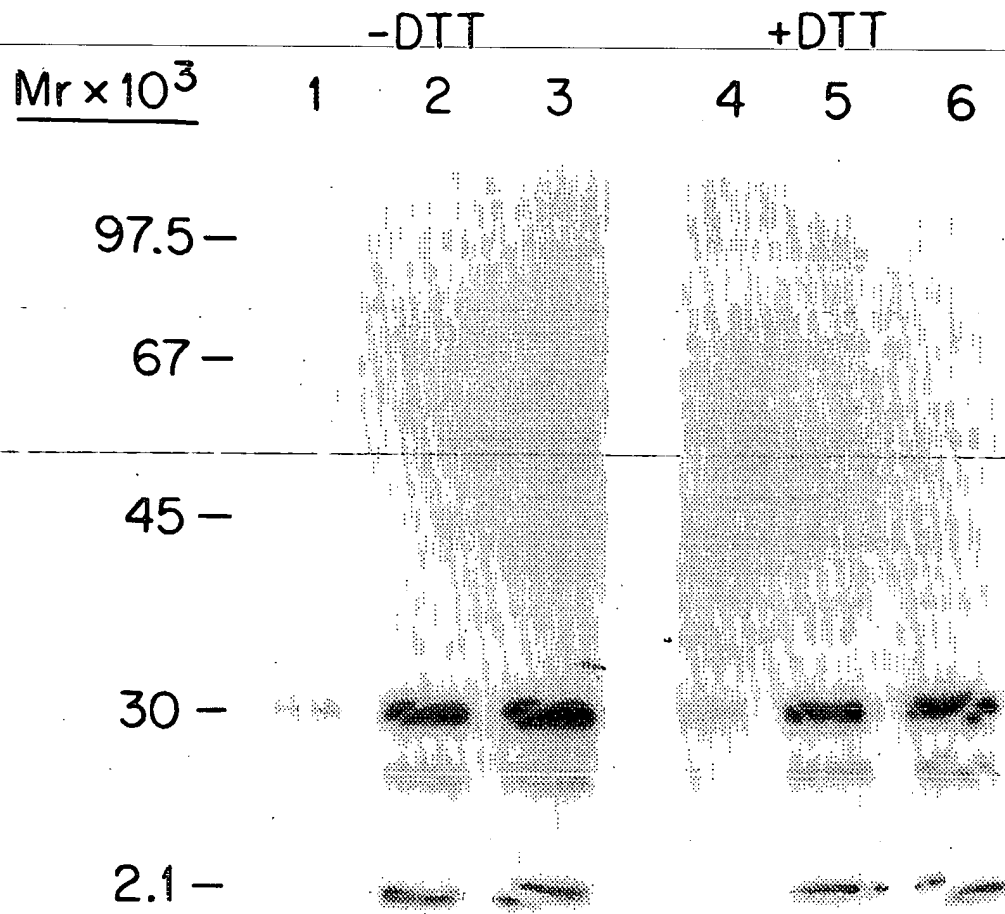


Fig. 4: SDS-PAGE analysis of C1q-bound ^{125}I -C1q INH. Solid phase binding assay was performed as described in legend to Figure 3, and the bound radioactivity eluted by addition of 50 μl SDS-PAGE sample buffer and the eluted solution analyzed by SDS-PAGE (10%) and autoradiography. Lane 1 is radioactivity bound to BSA whereas lanes 2 and 3 are those bound to C1q and gC1q respectively. The pattern is repeated in lanes 4-6 under reducing conditions.

minor bands with molecular weights of 28 kDa and 21 kDa were noted. This is consistent with previous work from our laboratory¹⁵ which showed that the major C1q binding activity could be isolated on HPLC using TSK gel filtration column. Radiolabeling of this activity and analysis by SDS-PAGE and autoradiography revealed this species to be composed of a single chain with a molecular weight of approximately 30 kDa. This 30 kDa species probably represents the proteoglycan core protein although no experimental evidence is

available to support this hypothesis. Recently, *Krumdieck et al.*¹⁶ showed that decorin, a small collagen-binding dermatan sulfate proteoglycan bound C1q at physiologic ionic strength and pH. This binding occurred primarily through a 36 kDa core protein of decorin to both the collagenous domain and the globular "heads" of C1q. Because the globular "heads" of C1q and the collagen "tails" share a short sequence of amino acids these authors postulated the junction of these two regions to be the binding site for decorin¹⁶.

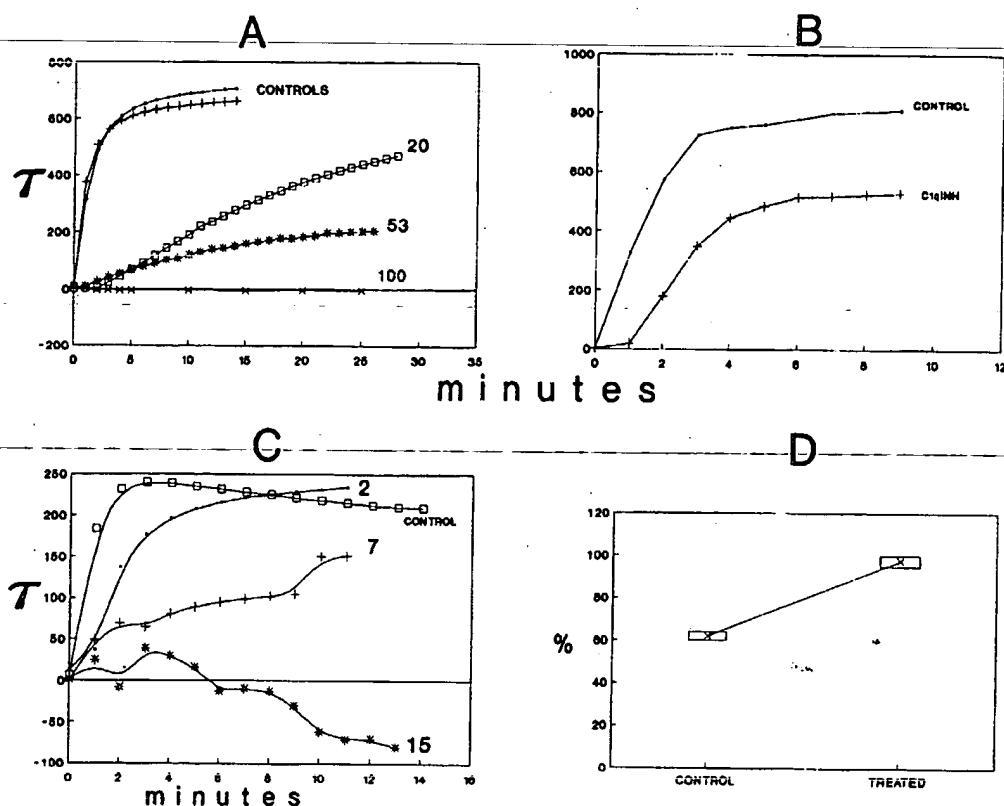


Fig. 5: Anticoagulant effects against fibrin gelation by the C1q INH.

A: Concentration dependent inhibition of plasma fibrin gelation. To fresh citrated plasma from several donors, 20% v/v in phosphate buffer, pH 6.4, $\mu = 0.19$, 37° , was added human thrombin to a final concentration of 0.2 U/ml. The turbidity of the mixture was monitored in time at 350 nm and is shown as absorbance $\times 1000$. Test mixtures contained C1q INH at the three different concentrations shown, in $\mu\text{g/ml}$, on the right of each graph. Some visible gel formed with the 20 and 53 C1q INH/ml but no gel formed at 100 $\mu\text{g/ml}$, even after 180 minutes (not shown).

B: Effect on batroxobin induced gelation of fibrinogen (1 $\mu\text{M/L}$) added to afibrinogenemic plasma, 50% in buffer, pH 7.4, $\mu = 0.15$, 35° . A visible gel formed which was more readily dispersible relative to the control gel. The C1q INH concentration was 10 $\mu\text{g/ml}$.

C: Effect on the re-polymerization of fibrin monomer, final concentration 2.2 $\mu\text{M/L}$, by three different concentrations of C1q INH ($\mu\text{g/ml}$) shown on the right of each test graph. To a 1.5 $\mu\text{M/L}$ fibrinogen solution, buffer as in B above, fibrin was added and allowed to polymerize in the presence or absence of the indicated C1q INH concentrations.

D: Effect of chondroitinase treatment of C1q INH, 5 $\mu\text{g/ml}$, on its fibrin-directed anticoagulant property. Shown are clot absorbance (350 nm) values, $n = 2$, calculated as % of maxima of clots formed in the absence of C1q INH. Clots were formed with 1 $\mu\text{M/L}$ fibrinogen and thrombin, 0.1 U/ml, pH 7.4. Control: untreated C1q INH. Treated: aliquot from the same C1q INH preparation, which had been digested with (0.1 U/ml) chondroitinase ABC, 37° , overnight.

(b) Anticoagulant effect on fibrinogen

i) Plasma studies

In view of the evidence¹⁷ that circulating proteoglycans found in certain clinical disorders possess anticoagulant properties against the thrombin/fibrinogen interaction, we examined

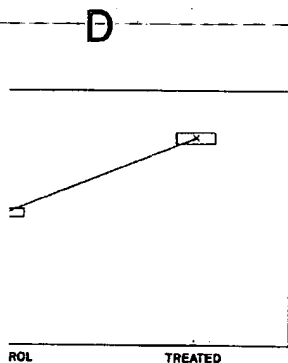
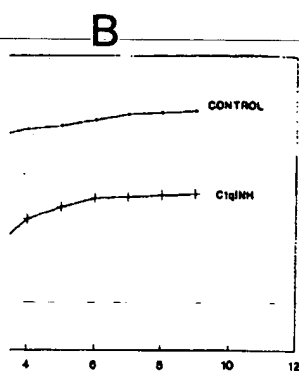
the possible effects of C1q INH on fibrinogen coagulability. The thrombin clotting time (TCT), a standard plasma clotting assay, was initially employed. To neutralize possible citrate anticoagulant effects the thrombin solution contained CaCl_2 to achieve a 67 mM/L final concentration. In plasma C1q INH induced a

concentration dependent TCT. For example, adding 100 $\mu\text{g/ml}$ prolonged the plasma control of 6.4 s to 104.9 s. When C1q INH was increased further the TCT was > 300 s ($n = 5$). This was observed when plasma clot turbidity was measured (Fig. 1A). In reported experiments, heparin and heparan sulfate glycosaminoglycans prolonged the plasma clotting time, the thrombin activity had no effect when plasma was treated with batroxobin, a snake venom which cleaves peptide A from fibrinogen. In contrast to such circulating plasma-batroxobin (or heparin) the TCT was prolonged by C1q INH. In a turbidimetric experiment (Fig. 1B), where fibrinogen added to plasma was clotted, the results showed delayed turbidity of the clot relative to the control.

In other experiments using batroxobin and C1q INH which permitted the formation of clots which formed were in sharp contrast to those reported. It was indicated that even when batroxobin was not adequately effective in the presence of XIIIa. Since control experiments showed that C1q INH has no effect on the catalytic activity, the clotting time was incomplete crosslink formation. In a series of normal gel or polymer studies, a series of experiments, trace amounts of radiolabeled fibrinogen were added to plasma. Coagulation was decreased to $\sim 20\%$ of control by increasing the C1q INH concentration (not shown).

ii) Isolated fibrinogen studies

Prolongation of the TCT of isolated fibrinogen solutions were observed when batroxobin or batroxobin. Increasing the concentration of batroxobin, e.g. 0.28 mM, had no demonstrable anticoagulant property



plasma from several donors, 20% v/v concentration of 0.2 U/ml. The turbidity, Test mixtures contained C1q INH the visible gel formed with the 20 and iogenemic plasma, 50% in buffer, e to the control gel. The C1q INH

by three different concentrations olution, buffer as in B above, fibrin 1 INH concentrations. anticoagulant property. Shown are ie absence of C1q INH. Clots were 1q INH. Treated: aliquot from the e ABC, 37°, overnight.

ects of C1q INH on fibrino- The thrombin clotting time plasma clotting assay, was . To neutralize possible ci- effects the thrombin soluti- 2 to achieve a 67 mM/L final plasma C1q INH induced a

concentration dependent prolongation of TCT. For example, addition of C1q INH to 40 $\mu\text{g/ml}$ prolonged the plasma TCT from the control of 6.4 s to 104.9 s ($n = 3$). When C1q INH was increased further to 100 $\mu\text{g/ml}$ the TCT was > 300 s ($n = 5$). Similar results emerged when plasma clot turbidity was measured (Fig. 1A). In reported clinical cases¹⁷ the proteoglycans prolonged the TCT by impairing the thrombin activity but characteristically had no effect when plasma was clotted by use of batroxobin, a snake venom protease which cleaves peptide A from fibrinogen. In sharp contrast to such circulating proteoglycans, the plasma batroxobin (or reptilase) clotting time was prolonged by C1q INH. A representative turbidimetric experiment is shown in Figure 1B, where fibrinogen added to afibrinogenic plasma was clotted with batroxobin. The results showed delayed onset and decreased turbidity of the clot relative to control.

In other experiments using amounts of C1q INH which permitted clot formation, the clots which formed were fully soluble in urea in sharp contrast to insoluble controls. This indicated that even when clots formed they were not adequately crosslinked by factor XIIIa. Since control experiments disclosed that C1q INH has no effect on the factor XIIIa catalytic activity, the clot solubility suggested incomplete crosslink formation owing to abnormal gel or polymer structure. In a different set of experiments, trace amounts of added radiolabeled fibrinogen was used to measure the amount of synerized clot following thrombin addition to plasma. Coagulable fibrinogen was decreased to $\sim 20\%$ of controls by progressively increasing the C1q INH concentrations (not shown).

ii) Isolated fibrinogen studies

Prolongation of the TCT was also shown when fibrinogen solutions were clotted with thrombin or batroxobin. Increased ionic strength, e.g. 0.28 mM, had no diminishing effect on the anticoagulant property of C1q INH. More-

over, the anticoagulant effect was completely abolished when C1q INH was digested with chondroitinase ABC (Fig. 1D) indicating its intact carbohydrate structure was required for the anticoagulant effect.

The possibility of contaminating Con A playing a role in the anticoagulant activity was excluded by two sets of experiments. In the first set, crude C1q INH tested prior to exposure to Con A displayed the anticoagulant effect (not shown). In the second set, a C1q INH isolate was exposed to solid phase anti-Con A IgG and tested before and after exposure by a turbidimetric assay. The results disclosed no significant differences before and after exposure to anti-Con A antibody.

A unique feature was the capacity of C1q INH to inhibit the re-polymerization of solubilized fibrin monomer. Here the key observation (Fig. 1C) was that its inhibition could not be influenced by the presence of fibrinogen so long as C1q INH was in molar excess. That is to say, premixed fibrinogen and C1q INH solutions, with the latter in at least several fold molar excess, did not diminish the inhibitory capacity of C1q INH against fibrin polymerization. This has implications on the mechanism of inhibition by C1q INH and is further considered in the discussion section.

A series of binding investigations reported elsewhere¹⁸ are briefly summarized here. These disclosed binding of radiolabeled C1q INH to fibrin clots formed in plasma and to microplate immobilized monomeric fibrinogen and fibrin. Moreover, cleaved fibrinopeptides were tightly bound by C1q INH. Consistent with this observation, C1q INH binding to the central or E fibrinogen domain was abolished when the fibrinopeptides had been cleaved, and this was demonstrated with isolated plasmonic fragment forms of this domain. Moreover, its binding to the outer domains, also shown by isolated fragment forms, appears to account for the observed binding to both monomeric fibrinogen and to fibrin. However, invariably higher amounts of C1q INH were bound by

fibrinogen than by monomeric fibrin, and this can be explained by the lack of binding to the fibrin central domain (i.e. lacking its fibrinopeptides).

Discussion

The C1q INH, which is a chondroitin 4-sulfate proteoglycan has the ability to interact with C1q. Although this interaction is enhanced by low ionic strength conditions, significant binding does occur even at physiologic ionic strength conditions suggesting that a substantial interaction may occur at sites where both molecules are generated in free form. Such interaction may in turn generate complexes which may participate in an ongoing inflammatory process by generating low level C1 activation. In both SLE and RA there is evidence that the level of glycosaminoglycans such as chondroitin sulfates and heparan sulfates increases and may reflect disturbances in immunoregulation⁶.

The mechanism of the C1q INH/fibrinogen interaction appears to involve two complementary but presumably independent events. One event involves binding to the fibrinopeptides whose susceptible Arg-Gly bonds are no longer accessible to thrombin. Because cleaved peptides were always bound by C1q INH they could not be measured, but this conclusion is supported by experiments in which no radio-labeled C1q INH could be released by thrombin from monomeric fibrinogen. Another event involves binding to the outer domain resulting in impaired polymerization of fibrin. The polymerization impairment appears to result from steric hindrance, for fibrinogen still inhibits fibrin polymerization in the presence of C1q INH excess. Also, the two inhibitors, C1q INH and fibrinogen, display additive inhibition against fibrin. In fact, the calculated slopes of inhibition of C1q INH in the presence and absence of fibrinogen were completely parallel (not shown); this indicated that: (a) fibrinogen was able to bind to fibrin E even though its D domain contained bound C1q

INH, and (b) the site(s) of C1q INH binding on-fibrinogen-D were other than those participating in polymerization.

As to the C1q INH epitope(s) which interacts with fibrinogen our results imply two general characteristics. One is that the epitope(s) is clearly distinct from that interacting with C1q. This was indicated by the fact that pre-incubation of C1q INH with C1q did not diminish its anticoagulant effect. Also, a polyclonal antibody which neutralized the C1q INH effect against C1q had no appreciable effect on its anticoagulant property. A second characteristic is implied by the stoichiometry of the C1q INH/fibrinogen interaction. That is to say, when the molar C1q INH concentrations were less than those of fibrinogen appreciable anticoagulant effect could still be demonstrated. This along with a clear binding to two different fibrinogen domains implies that at least two epitopes on C1q INH are capable of interacting with fibrinogen. Moreover, when one was occupied with one fibrinogen molecule the second epitope was still able to interact with another fibrinogen molecule. Additionally, abolition of the interaction by chondroitinase clearly implicates the carbohydrate structure of C1q INH.

Evidence for the selectivity of the interaction in plasma can be marshalled from the plasma clot binding results, the plasma fibrinogen coagulability studies, and the comparative binding in microplate assays showing C1q INH binds in higher amounts to C1q and to fibrinogen than to albumin or IgG. The nature of the interaction is unclear. Neither calcium excess nor high ionic strength (0.28, not shown) could diminish or enhance the anticoagulant effect.

Acknowledgements

The studies presented here were supported in part by grants CA-41047 from the National Cancer Institute and 900696 from the National Center of the American Heart Associa-

tion. The authors wish to thank Dr. Reid (University of Oxford) for a generous gift of cC1q and for skillful technical assistance. Dr. Veprek for excellent secretarial assistance.

References

- 1 Conradie, J. D., Volanakis, A. E. (1975) Evidence for a serum chondroitinase. *Biochemistry* 12, 967-971.
- 2 Yonemasu, K. & Stroud, R. (1975) A purification method for preparation of sera and for biochemical studies. *J. Biol. Chem.* 250, 304-313.
- 3 Ghebrehewet, B. (1981) C1q INH: Functional properties and interaction with lymphocyte membrane-associated C1q. *J. Immunol.* 126, 1837.
- 4 Silvestri, L., Baker, J. R., R. (1981) The C1q inhibitor in plasma. *J. Biol. Chem.* 256, 1132-1134.
- 5 Ghebrehewet, B. & Müller-Lissner, S. (1981) The C1q inhibitor in plasma. *J. Biol. Chem.* 256, 1132-1134.
- 6 Friman, C., Nordström, D. & Nordström, D. (1981) Glycosaminoglycans in synovial fluid. *J. Rheumatol.* 14, 1132-1134.
- 7 Mancini, G., Carbonara, A. (1965) Immunochemical quantitation of single radial immunodiffusion. *Immunochemistry* 2, 235-254.
- 8 Ouchterlony, O. (1964) *Gel Diffusion in Immunology*. J. Davis Philadelphia.

site(s) of C1q INH binding were other than those participating.

The epitope(s) which interacts with fibrinogen results imply two general conclusions. One is that the epitope(s) is on the C1q molecule interacting with C1q. This is supported by the fact that pre-incubation of fibrinogen with C1q did not diminish the effect. Also, a polyclonal antiserum against the C1q INH effect had no appreciable effect on its activity. A second characteristic of the interaction is the stoichiometry of the interaction. That is to say, at low C1q INH concentrations, the inhibition of fibrinogen appreciable could still be demonstrated. This implies that at least two binding sites are capable of interaction. Moreover, when one was able to interact with the fibrinogen molecule the other is still able to interact with another molecule. Additionally, the interaction by chondroitinase does not alter the carbohydrate structure of the C1q INH.

The selectivity of the interaction was established from the plasma, the plasma fibrinogen coagulation and the comparative binding assays showing C1q INH binding to C1q and to fibrinogen or IgG. The nature of the interaction is unclear. Neither calcium enhancement (0.28, not shown) nor enhancement of the anticoagulant activity.

ts

The work here was supported in part by grant 41047 from the National Institutes of Health and 900696 from the National American Heart Association.

The authors wish to thank Dr. K. B. M. Reid (University of Oxford, U.K.) for his generous gift of cC1q and gC1q, Yuqun Hong for skillful technical assistance and Maureen Veprek for excellent secretarial work.

References

- 1 Conradie, J. D., Volanakis, J. E. & Stroud, R. M. (1975) Evidence for a serum inhibitor of C1q. *Immunochimistry* 12, 967-971.
- 2 Yonemasu, K. & Stroud, R. M. (1991) C1q: rapid purification method for preparation of monospecific antisera and for biochemical studies. *J. Immunol.* 106, 304-313.
- 3 Ghebrehewet, B. (1981) C1q inhibitor (C1q INH): Functional properties and possible relationship to a lymphocyte membrane-associated C1q precipitin. *J. Immunol.* 126, 1837.
- 4 Silvestri, L., Baker, J. R., Roden, L. & Stroud, R. M. (1981) The C1q inhibitor in serum is a chondroitin 4-sulfate proteoglycan. *J. Biol. Chem.* 256, 7383-7387.
- 5 Ghebrehewet, B. & Müller-Eberhard, H. J. (1987) Lysis of C1q-coated chicken erythrocytes by human lymphoblastoid cell lines. *J. Immunol.* 120, 27-32.
- 6 Friman, C., Nordström, D. & Eronen, I. (1987) Plasma glycoaminoglycans in systemic lupus erythematosus. *J. Rheumatol.* 14, 1132-1134.
- 7 Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochimistry* 2, 235-254.
- 8 Ouchterlony, O. (1964) Gel diffusion techniques. In: *Immunological Methods* (J. F. Ackroyd, Ed.), p. 55. Davis Philadelphia.
- 9 Kolb, W. P., Kolb, L. M. & Podack, E. R. (1979) C1q: isolation from human serum in high yield by affinity chromatography and development of a highly sensitive hemolytic assay. *J. Immunol.* 122, 2103-2111.
- 10 Newton, D. J., Scott, J. E. & Whiteman, P. (1974) The estimation of acid glycosaminoglycan-Alcian blue complexes eluted from electrophoretic strips. *Anal. Biochem.* 62, 268-273.
- 11 Bartold, P. M. & Page, R. C. (1985) A microdetermination method for assaying glycoaminoglycans and proteoglycans. *Anal. Biochem.* 150, 320-324.
- 12 Høök, M., Kjellén, L., Johansson, S. & Robinson, J. (1984) Cell surface glycosaminoglycans. *Annu. Rev. Biochem.* 54, 847-869.
- 13 Ghebrehewet, B. & Humberger, M. (1982) Purification and partial characterization of a C1q inhibitor from the membranes of human peripheral blood lymphocytes. *J. Immunol.* 129, 157-162.
- 14 Ghebrehewet, B., Silvestri, L. & McDevitt, C. (1984) Identification of the Raji cell membrane-derived C1q inhibitor as a receptor for human C1q. Purification and immunochemical characterization. *J. Exp. Med.* 160, 1375-1389.
- 15 Bossone, S. A. & Ghebrehewet, B. (1987) Further characterization of C1q inhibitor. *Complement* 123, 137 (abstr).
- 16 Krumdieck, R., Høök, M., Rosenberg, L. C. & Volanakis, J. E. (1992) The proteoglycan decorin binds to C1q and inhibits the activity of the C1 complex. *J. Immunol.* 149, 3695-3701.
- 17 Liebman, H. A., Cömenzo, R., Aleen, S. T., Dilorio, J. M. (1991) A glycosaminoglycan inhibitor of thrombin: A new mechanism for abnormal hemostatic assays in cancer. *Am. J. Hematol.* 38, 24.
- 18 Galanakis, D. K., Ghebrehewet, B. (1992) Anticoagulant mechanisms of the plasma C1q inhibitor: Evidence of binding to D and E fibrinogen domains. *Circulation* 86 (suppl.), 1-738.

STIC-ILL

QP501. B47
mic

From: Romeo, David
Sent: Monday, June 04, 2001 1:24 PM
To: STIC-ILL
Subject: 09503421 Kishore U; Sontheimer R D; Sastry K N; Zaner K S; Zappi E G; Hughes G R;

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
- Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Kishore U; Sontheimer R D; Sastry K N; Zaner K S; Zappi E G; Hughes G R;
Khamashta M A; Strong P; Reid K B; Eggleton P
TI Release of calreticulin from neutrophils may alter Clq-mediated immune
functions.
SO BIOCHEMICAL JOURNAL, (1997 Mar 1) 322 (Pt 2) 543-50.
Journal code: 9YO; 2984726R. ISSN: 0264-6021.
AN 97218114

Release of calreticulin from neutrophils may alter C1q-mediated immune functions

Uday KISHORE*, Richard D. SONTHEIMER†, Kedar N. SASTRY‡, Ken S. ZANER‡, Eugene G. ZAPPI†, Graham R. V. HUGHES§, Munther A. KHAMASHTA§, Peter STRONG*, Kenneth B. M. REID* and Paul ELLINGTON¶

*MRC Immunochimistry Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K., †Department of Dermatology & Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A., ‡Department of Pathology, Boston University School of Medicine, Boston, MA, U.S.A., and §The Rayne Institute, St. Thomas' Hospital, London, U.K.

Calreticulin is an abundant intracellular protein which is involved in a number of cellular functions. During cytomegalovirus infection, as well as inflammatory episodes in autoimmune disease, calreticulin can be released from cells and detected in the circulation, where it may act as an immunodominant autoantigen in diseases such as systemic lupus erythematosus. Calreticulin is known to bind to the molecules of innate immunity, such as C1q, the first subcomponent of complement. However, the functional implications of C1q-calreticulin interactions are unknown. In the present study we sought to investigate, in greater detail, the interaction between these two proteins following the release of calreticulin from neutrophils upon stimulation. In order to pinpoint the regions of interaction, recombinant calreticulin and

its discrete domains (N-, P- and C-domains) were produced in *Escherichia coli*. Both the N- and P-domains of calreticulin were shown to bind to the globular head regions of C1q. Calreticulin also appeared to alter C1q-mediated immune functions. Binding of calreticulin to C1q inhibited haemolysis of IgM-sensitized erythrocytes. Both the N- and P-domains of calreticulin were found to contain sites involved in the inhibition of C1q-induced haemolysis. Full-length calreticulin, and its N- and P-domains, were also able to reduce the C1q-dependent binding of immune complexes to neutrophils. We conclude that calreticulin, once released from neutrophils during inflammation, may not only induce an antigenic reaction, but, under defined conditions, may also interfere with C1q-mediated inflammatory processes.

INTRODUCTION

Calreticulin is an autoantigen of clinical interest, because antibodies against it are found in many patients suffering from lupus disorders and Sjogren's syndrome [1,2]. Human calreticulin has been cloned and found to be 417 amino acids in length, with a calculated molecular mass of 60 kDa [3]. Calreticulin has been found in nearly all eukaryotic cell types with the exception of erythrocytes, at concentrations ranging from 20 to 500 µg/g of tissue. The precise biological roles of calreticulin are still under discussion, but the protein, by virtue of its C-terminal KDEL sequence, is known to be found associated with the lumen of the endoplasmic reticulum (ER), where it is believed to function as a high-capacity Ca²⁺ storage and regulatory protein [4]. In leucocytes, the ER is very poorly defined, and 'calreticulin-like' proteins are present in discrete subcellular compartments other than the ER. For example, HL-60 cells possess a 60 kDa Ca²⁺-binding protein in 'calciosomes' with an N-terminal sequence that shares 93% identity with calreticulin over the first 15 amino acid residues [5], and calreticulin has also been identified in discrete secretory granules of cytolytic T-lymphocytes [6]. Calreticulin has also been detected at low levels in the plasma of normal individuals, probably originating from cells [7]. It is also overexpressed on the surface of lung fibroblasts in response to cytomegalovirus infection [8].

Several studies have indicated that calreticulin shares 50–60% amino acid identity with proteins found in the human parasites *Onchocerca volvulus* [9] and *Schistosoma mansoni* [10], which may help to explain why calreticulin is a target for autoimmunity.

What is less clear is whether, on its release from cells, calreticulin has a physiological/pathological role. In a previous study we showed that C1q, a subunit of the first component of complement (C1), which provides the initial trigger for the activation of the classical complement cascade, binds to calreticulin [11]. Potentially, the association of calreticulin with C1q may interfere with complement activation. In classical pathway complement activation, the C-terminal globular head region of C1q binds to the CH₂ domains of immune complex (IC)-fixed immunoglobulin [12]. When C1q-IC interactions are formed *in vivo*, the subcomponents C1r and C1s become activated and initiate complement activity. The cascade is regulated in part by C1 inhibitor, which binds to activated C1r and C1s, thereby inactivating these enzymes, which become dissociated from C1q-IC. In this process, complement is consumed during IC formation, which is reflected in patients with systemic lupus erythematosus (SLE), who have decreased complement levels [13]. Complement activation is an inflammatory process, but it is also involved in the prevention of formation of large insoluble ICs, which might cause tissue injury [14]. In SLE, it appears that both the prevention of antigen-antibody complexes and the consequent clearance of ICs at sites of deposition are impaired.

Leucocytes contain a large intracellular store of calreticulin. In the present study we show that calreticulin is released from activated neutrophils, and thus has the potential to associate with serum proteins. Employing native and recombinant calreticulin, and the individual N-, P- and C-domains of this protein, we have attempted to locate the binding domain for C1q-calreticulin interactions and examine whether calreticulin

Abbreviations used: C1qR, collectin receptor; DGVB⁺⁺, isotonic Veronal buffered saline containing 0.1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% (w/v) gelatin and 1% (w/v) glucose; EA, sensitized sheep erythrocytes; ER, endoplasmic reticulum; FMLP, formylmethionyl-leucyl-phenylalanine; HAGG, heat-aggregated human IgG; IC, immune complex; MBP, maltose-binding protein; PMA, phorbol 12-myristate 13-acetate; SLE, systemic lupus erythematosus.

¶ To whom correspondence should be addressed.

interferes with classical complement activation, which is important for ICsubilization and clearance. In particular, the N-terminal portion of calreticulin was found to (a) reduce C1q-dependent IC binding to neutrophils, and (b) interfere with C1q-dependent haemolysis of sensitized erythrocytes in a dose-dependent manner. These results suggest that the release of calreticulin from leucocytes during inflammation may impede some of the functions of the classical complement pathway that are necessary for IC clearance.

MATERIALS AND METHODS

Antibodies and reagents

Affinity-purified rabbit antibodies against recombinant human calreticulin expressed in the baculovirus system [15] and against synthetic peptides corresponding to N-terminal residues 7–28 and the final C-terminal residues (399–417) of human calreticulin were prepared as previously described [3]. Murine anti-(human C1q) was purchased from Quidel®, San Diego, CA, U.S.A. FITC-labelled rabbit and mouse anti-human IgG were obtained from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A.). Dulbecco's PBS (with and without calcium and magnesium), formylmethionyl-leucyl-phenylalanine (FMLP) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The expression vector pMal-c2, amylose resin and Factor Xa protease were obtained from New England Biolabs (Beverly, MA, U.S.A.).

Serum samples

Blood samples were drawn by venepuncture from healthy consenting adults in accordance with the Declaration of Helsinki of the World Medical Association. Serum was isolated from the blood by centrifugation.

Isolation and preparation of neutrophils

Human neutrophils were isolated from whole blood taken from normal donors by a one-step isolation procedure using Polymorphprep® (Nycomed), as previously described [16]. The isolated cells (> 95% pure) were then suspended in PBS, pH 7.4, without calcium or magnesium, and maintained at 4 °C. For the experiments requiring stimulated neutrophils, cells were pre-warmed to 37 °C for 5 min before stimulation with either FMLP (0.1 µM) or PMA (100 ng/ml) for 15 min. The cells were then washed and resuspended in the appropriate buffer. Neutrophils, before and after stimulation, were stained with Trypan Blue for 10 min and then examined by light microscopy to monitor the integrity of the plasma membrane.

Purification of native C1q and calreticulin

Haemolytically active C1q was isolated from human serum as described by Reid [17] and its purity assessed by SDS/PAGE on a 5–20% (w/v) polyacrylamide gel under reducing conditions. C1q was subjected to linear sucrose gradient centrifugation (5–31%), which confirmed the presence of a single non-aggregated protein. As a precaution, C1q was centrifuged at 4 °C at 14000 g for 15 min immediately prior to use, to eliminate any aggregates which may have formed during storage. Globular heads of C1q were prepared by digestion of C1q with collagenase purified from *Achromobacter iophagus* [18]. Similarly, collagen tails of C1q were prepared by digestion of C1q with pepsin [19].

The recombinant globular head of the B chain of C1q was produced as described [20]. Native calreticulin was purified using the published method [21].

Construction of plasmids expressing different domains of calreticulin

For the expression and isolation of recombinant proteins, a maltose-binding protein (MBP) fusion system was used consisting of the pMal-c2 plasmid [22]. This expression vector encodes *E. coli* MBP followed by the Factor Xa cleavage site, a unique blunt-end *XmnI* cloning site at the 5' end and an *EcoRI* site at the 3' end. For the purposes of this study, we expressed the protein in three domains: the N-terminal domain (N-domain; amino acids 1–181), the proline-rich domain (P-domain; amino acids 182–292) and the C-terminal domain (C-domain; amino acids 293–417). The putative domain organization was based on the known intron/exon structure [4]. PCR was used to amplify open reading frames encoding full-length calreticulin and the N-, P- and C-domains. A 1.9 kb *SacI* fragment containing cDNA for human calreticulin was used as a template. The following six terminal primers were designed: FP-ND, 5' GCCGTCGCCGTCGCCCCGGGAGCCCCGCGTCTAC 3' (34-mer); RP-ND, 5' CCCCCCAATTCCTATTCCAAGGAGCCGGA 3' (30-mer); FP-PD, 5' GGCTCCTTGGAAACCCGGGGACGATTGG 3' (27-mer); RP-PD, 5' CCCCCGAATTCCTAGGCA-TAGATACTGGG 3' (30-mer); FP-CD, 5' CCCAGTATCTA-TGCCCGGGTATGATAAC 3' (27-mer); RP-CD, 5' CCCCC-CAGGAATTCCTACAGCTCGTCCTTGGG 3' (33-mer). The PCR products corresponding to full-length calreticulin and the N-, P- and C-domains (ND, PD and CD refer to N-, P- and C-domains respectively) were cleaved with *SmaI* and *EcoRI* and subcloned into the pMal-c2 vector restriction-digested with *XmnI* and *EcoRI*. The recombinant plasmids containing the N-, P- and C-domains of human calreticulin were designated pN-CRT, pP-CRT and pC-CRT respectively.

Expression and purification of recombinant proteins

E. coli BL21 harbouring various constructs were grown in Luria broth containing ampicillin (50 µg/ml) at 37 °C with vigorous aeration to an A_{490} of 0.6. Isopropyl β -D-thiogalactoside (NOVA Biochem) was added to a final concentration of 0.4 mM to induce the P_{lac} promoter in the expression vector. The cells were shaken under these conditions for 3 h and harvested. Cells from 1 litre of culture (3 g of cell pellet) were suspended in 50 ml of lysis buffer [20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.25% (v/v) Tween 20, 1 mM EGTA, 1 mM EDTA and 5% (v/v) glycerol]. Lysozyme and PMSF (Sigma) were added to final concentrations of 100 µg/ml and 0.1 mM respectively. All subsequent steps of purification were carried out at 4 °C. The cell suspension was incubated with lysis buffer over ice for 30 min and the lysate was then sonicated at 60 Hz for 30 s bursts with intervals of 1 min (15 cycles) to shear the bacterial chromosomal DNA. After centrifugation at 14000 g for 30 min at 4 °C, the supernatant was collected and diluted 5-fold with column buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.25% (v/v) Tween 20, 1 mM EDTA and 5% (v/v) glycerol], and loaded on to an amylose resin column (50 ml bed volume; New England Biolabs) equilibrated with the same column buffer. The column was washed successively with 3 bed volumes of column buffer, 5 bed volumes of column buffer without Tween 20 and 5 bed volumes of Factor Xa buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl₂ and 5% (v/v) glycerol]. The fusion protein, in each case, was eluted with 100 ml of Factor Xa buffer containing 10 mM maltose. The peak fractions were pooled and Factor Xa

(1 unit/ μ l; New England Biolabs) was added (1 unit f Factor Xa per 100 μ g of fusion protein) to release the domains from the fusion partner, wherever required.

Indirect Immunofluorescence

Neutrophils were incubated for 30 min with 0.1 μ M FMLP or 100 ng/ml PMA in PBS. Then 1×10^6 cells in 10 μ l aliquots were allowed to adhere to glass slides for 30 min at 37 °C in a humidified atmosphere. Slides were washed extensively with PBS and fixed in 2% (v/v) paraformaldehyde for 1 h at 4 °C. Cells were washed and then incubated in a 1:20 dilution of preimmune serum or anti-calreticulin antibodies for 1 h. The secondary antibody (1:50 dilution) was added [anti-rabbit IgG (Fab)₂ conjugated to rhodamine; Tago Immunologicals, Burlingame, CA, U.S.A.]. Following a 1 h incubation, the slides were mounted in Cytoseal 60 mounting medium (Stephens Scientific, Riverdale, NJ, U.S.A.). The cells were examined using a Nikon inverted microscope Diaphot TMP employing epifluorescence microscopy under oil immersion at 1000 \times magnification. A Nikon N6000 camera containing Kodak Tri-X pan 400 ASA black-and-white film was used to photograph the rhodamine-stained cells.

Measurement of calreticulin levels by ELISA

Cell suspension media from control and stimulated cells were collected by gently pelleting the cells at 325 g for 1 min. The supernatant was spun at 16000 g for 20 min to remove any particulate matter, and then coated on to microtitre plate wells. BSA and calreticulin purified from Wil-2 cells (150 ng/well) were used as negative and positive control proteins respectively. All samples were prepared in sodium carbonate buffer (pH 9.6) at the required concentration and allowed to bind to the wells for 14 h at 4 °C. Preimmune rabbit IgG acted as a non-specific immunoglobulin. Plates were washed in PBS/Tween 20 and non-specific binding sites were blocked with 5% (w/v) fat-free milk/100 mM glycine. Immunoaffinity-purified anti-calreticulin antibody (1:200 dilution) was added to each well for 1 h at 37 °C, followed by horseradish peroxidase-conjugated anti-rabbit polyclonal IgG (1:1000 dilution). 3,3',5,5'-Tetramethyl benzidine-peroxidase substrate was added to each well and the colour was allowed to develop for 20 min. The reaction was terminated by adding 150 μ l of 2 M H₂SO₄ and the A₄₅₀ of the plate was read.

Assay of C1q-dependent haemolysis

C1q-deficient serum (Sigma) was diluted 1:40 times in DGVB⁺⁺ [isotonic Veronal buffered saline containing 0.1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% (w/v) gelatin and 1% (w/v) glucose], and various concentrations of purified C1q were added in a final volume of 100 μ l prior to incubation at 37 °C for 1 h. C1q haemolytic assays were performed by adding 100 μ l of sensitized sheep erythrocytes (EA) (10⁷ cells) in DGVB⁺⁺ and incubating for a further 1 h. The reaction was stopped by transferring the tubes to an ice bath and adding 0.6 ml of ice-cold DGVB⁺⁺. The unlysed cells were pelleted by centrifugation and the A₄₁₂ values of 100 μ l aliquots of supernatant were read to monitor haemoglobin release. The amount of C1q required to cause 75–85% haemolysis was determined.

Various samples for assay of C1q-dependent haemolysis were prepared as follows. Highly purified C1q (3 μ g) was added to various concentrations of native or recombinant calreticulin or its domains (0–4 μ g) in a final volume of 100 μ l of DGVB⁺⁺ and incubated at 37 °C for 60 min. To each sample was added 100 μ l of EA (10⁷ cells in total), followed by incubation at 37 °C for an additional 1 h. The unlysed cells were pelleted and the amount of

haemoglobin released was determined spectrophotometrically at 412 nm. Total haemolysis was the amount of haemoglobin released upon cell lysis with water. C1q-dependent haemolytic activity was expressed as a percentage of total haemolysis. *E. coli* MBP was used as a control protein.

Binding of heat-aggregated human IgG (HAGG) to neutrophils in the presence of C1q and calreticulin

IgG was isolated from human serum as described previously [23]. Purified IgG (18–20 mg/ml) in water was heated to 63 °C for 30 min. The immunoglobulin was centrifuged at 14000 g for 20 min to remove insoluble precipitates. The HAGG was then adjusted to a concentration of 10 mg/ml and stored at 4 °C. C1q (30 μ g/ml) was incubated with and without calreticulin (5 μ g) or its domains for 1 h at 37 °C, and then incubated again with HAGG at a final concentration of 50 μ g/ml for an additional 1 h at 37 °C. Aliquots (100 μ l) of the various preparations were then incubated with 5×10^6 neutrophils in PBS for 30 min at 4 °C. After washing, the binding of HAGG, HAGG–C1q and HAGG–C1q–calreticulin preparations to neutrophils was determined by incubating the cells with rabbit anti-human IgG (1:50 dilution) followed by goat anti-rabbit FITC-conjugated antibody (1:50 dilution). Analysis was performed on 5000 cells by flow cytometry employing Lysis II software version 1.1 (Becton Dickinson) and the mean fluorescence intensity was presented.

RESULTS

Release of calreticulin from activated neutrophils and detection in serum

To study the release of calreticulin from activated neutrophils, cells were stimulated with 0.1 μ M FMLP or 100 ng/ml PMA or left untreated for 15 min at 37 °C. Anti-calreticulin antibody against the full-length protein was used to detect extracellular release of calreticulin into the culture medium from stimulated neutrophils from seven normal subjects. ELISA revealed the presence of calreticulin in different amounts (Figure 1, part i). The concentration of calreticulin detected in the extracellular media from 1×10^6 non-stimulated and PMA-stimulated cells incubated at 37 °C ranged between 1 and 4 ng, but a 2–3-fold increase was observed after stimulation with FMLP, with values ranging between 2 and 12 ng/10⁶ cells ($n = 7$, $P < 0.05$). To investigate further whether the stimulation by FMLP resulted in the release of calreticulin into the medium, immunofluorescence microscopy was used. Cells (10⁷/ml) in PBS from healthy donors were stimulated under the same conditions, then probed with anti-calreticulin and goat anti-rabbit antisera conjugated to rhodamine to analyse calreticulin surface localization and expression in non-permeabilized neutrophils. As shown in Figure 1, part (ii) (panels A and C), non-stimulated and PMA-stimulated cells showed evidence of surface staining with anti-calreticulin antibodies. However, a striking difference was observed for FMLP-stimulated cells (Figure 1, part ii, panel B); the calreticulin staining on these cells, as well as being evident on or near the surface, was also present extracellularly. As a control for non-specific staining, neutrophils were stimulated in a similar fashion and probed with rabbit preimmune antiserum, which resulted in very low levels of fluorescence (results not shown).

In order to determine if significant levels of calreticulin could be detected in the sera of healthy subjects, calreticulin was measured in 23 control sera. As expected, only a small number of sera samples had measurable levels of calreticulin, with a mean value of 1.94 μ g/ml, whereas in the overall majority of samples (18 out of 23), calreticulin was not detected. The C1q levels in the

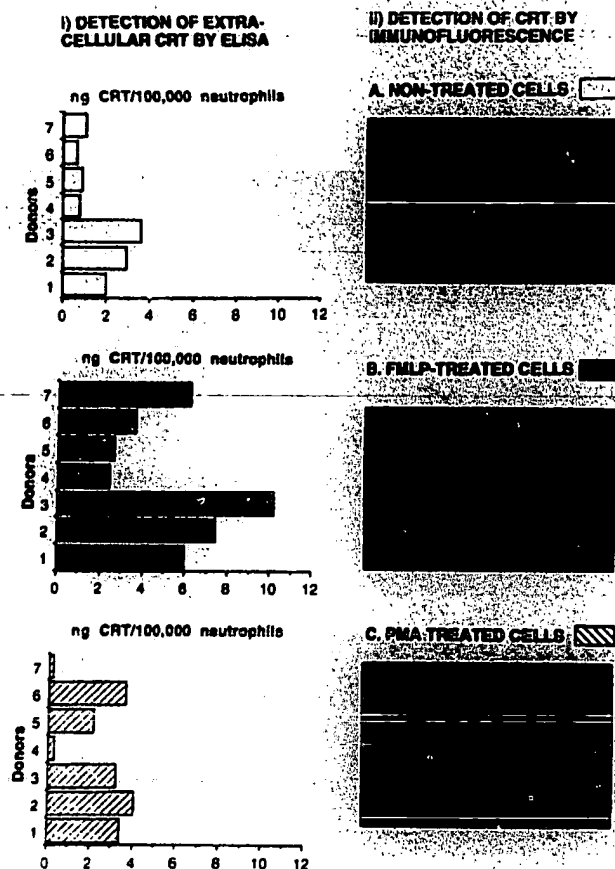


Figure 1 Release of calreticulin from stimulated neutrophils

Part (i): ELISA analysis of calreticulin released into the cell suspension buffer from non-stimulated and stimulated neutrophils from seven normal donors. Neutrophils ($10^5/100 \mu\text{l}$) were incubated with PBS, $0.1 \mu\text{M}$ FMLP or 100 ng/ml PMA for 15 min at 37°C . The cells were pelleted at low speed and the cell suspension media were assayed for calreticulin (CRT) by a standard ELISA method. The A_{450} was read and the amount of calreticulin calculated from a standard curve obtained with purified calreticulin. Part (ii): indirect immunofluorescence of calreticulin localization in neutrophils under non-stimulated (A), FMLP-stimulated (B) and PMA-stimulated (C) conditions. Magnification approx. $\times 1500$.

same subjects were also measured and were found to be normal ($56.4 \pm 14.9 \mu\text{g/ml}$; mean \pm S.D.).

Recombinant production of human calreticulin and its various domains in *E. coli*

Human calreticulin was expressed as three distinct domains. The expression and purification of each fusion protein containing *E. coli* MBP was performed as described previously [24]. Calreticulin migrated with an apparent molecular mass of 60 kDa on SDS/PAGE, although the predicted molecular mass is 46 kDa . The MBP-calreticulin fusion protein also migrated with the same apparent mobility as a major band of 97 kDa (Figure 2, lane 5). The three domains of calreticulin, N-, P- and C- (Figure 2, lanes 2–4 respectively), of predicted molecular masses of 20 , 16 and 16 kDa respectively, migrated as bands of approx. 60 kDa when fused to MBP (42 kDa). The full-length recombinant protein and the three domains reacted with polyclonal rabbit anti-(human calreticulin) antiserum in Western blot analysis

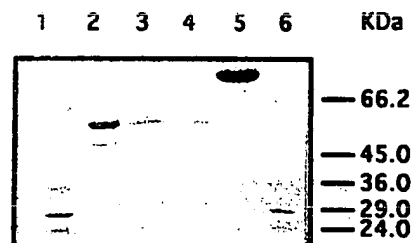


Figure 2 Purification of recombinant calreticulin and its N-, P- and C-domains

Lanes 1 and 6 contain the molecular mass standards shown (kDa). Lane 5 contains purified full-length calreticulin fused to MBP. Lanes 2–4 contain the N-, P- and C-domains respectively of calreticulin fused to MBP, after purification from an amylose resin column. All the samples were run on SDS/PAGE (10% (w/v) gel) after reduction of the disulphide bonds.

Table 1 C1q binds predominantly to the N-terminal half of calreticulin

Microtitre wells were coated with 0.25 , 1.0 or $4.0 \mu\text{g}$ of full-length calreticulin or its N-, P- or C-domains. MBP and BSA were used as control proteins. After washing three times and blocking with milk/PBS, 125 ng of C1q ($50 \mu\text{l}$) was added to each well in the presence of normal or half physiological salt concentrations for 2 h at 37°C . After additional washing, binding of C1q to each protein was detected by probing with rabbit anti-(human C1q) (1:5000 dilution), followed by anti-rabbit IgG (1:4000 dilution) conjugated to horseradish peroxidase, each for 1 h at 37°C . The ELISA plates were then developed and A_{450} readings taken. Results are means \pm S.D. of three experiments.

Amount of calreticulin or domain added/well (μg)	Binding (A_{450} units)			
	Native CRT	N-domain	P-domain	C-domain
Half physiological salt concentration				
4.0	0.89 ± 0.23	0.95 ± 0.22	0.43 ± 0.16	0.26 ± 0.12
1.0	0.78 ± 0.36	0.44 ± 0.09	0.16 ± 0.03	0.12 ± 0.09
0.25	0.43 ± 0.20	0.24 ± 0.24	0.15 ± 0.18	0.19 ± 0.22
Physiological salt concentration				
4.0	0.23 ± 0.14	0.37 ± 0.14	0.07 ± 0.03	0.04 ± 0.04
1.0	0.07 ± 0.06	0.17 ± 0.06	0.03 ± 0.05	0.02 ± 0.06
0.25	0.07 ± 0.06	0.05 ± 0.06	0.01 ± 0.06	0.02 ± 0.09

(results not shown). MBP alone did not react with anti-calreticulin antiserum.

Identification of the region of calreticulin that binds to C1q

As calreticulin is known to bind to C1q, it was felt important to localize further the region of calreticulin that binds to C1q. Therefore various amounts of the N-, P- and C-domains of calreticulin (4 , 1 and $0.250 \mu\text{g}$) were bound on ELISA plates overnight. A polyclonal antibody against calreticulin was used to confirm the binding of each recombinant domain to the wells in a dose-dependent manner. The domains were then incubated with 125 ng of native C1q in PBS or half-strength PBS for 2 h at 37°C , followed by probing with rabbit anti-(human C1q) antibody (1:3000 dilution) and anti-rabbit antiserum conjugated to horseradish peroxidase. As shown in Table 1, C1q bound predominantly to the N-domain of calreticulin under physi-

Table 2 Calreticulin binding to different regions of C1q

Microtitre wells were coated with 0.25, 1.0 or 4.0 µg of C1q, the collagen tails of C1q, the globular heads of C1q or the recombinant globular head of the B chain of C1q (ghB). After washing three times and blocking with 1% (w/v) milk/glycine/PBS, 0.5 µg of native calreticulin was added to each well and incubated in normal and half-strength PBS for 2 h at 37 °C. After additional washing, calreticulin binding was determined by probing with rabbit anti-human calreticulin (1:4000 dilution, 2 h, 37 °C) and then analysed by ELISA and A_{450} values measured. Results are means \pm S.D. of triplicate experiments. MBP acted as a negative control protein.

Amount of C1q or fragments added/well (µg)	Binding (A_{450} units)				
	Native C1q	C1q tails	C1q heads	ghB	MBP
Half physiological salt concentration					
4.0	1.84 \pm 0.34	0.08 \pm 0.09	0.23 \pm 0.30	0.48 \pm 0.09	0.08 \pm 0.01
1.0	1.55 \pm 0.30	0.10 \pm 0.03	0.20 \pm 0.01	0.37 \pm 0.04	0.08 \pm 0.01
0.25	0.62 \pm 0.02	0.06 \pm 0.03	0.06 \pm 0.03	0.31 \pm 0.01	0.08 \pm 0.02
Physiological salt concentration					
4.0	1.15 \pm 0.06	0.08 \pm 0.01	0.43 \pm 0.16	0.26 \pm 0.12	0.06 \pm 0.03
1.0	0.65 \pm 0.06	0.08 \pm 0.01	0.16 \pm 0.03	0.12 \pm 0.09	0.07 \pm 0.02
0.25	0.29 \pm 0.09	0.07 \pm 0.00	0.15 \pm 0.18	0.19 \pm 0.22	0.09 \pm 0.08

logical salt conditions. When less stringent salt conditions were employed, C1q binding to the N-domain was enhanced, and even weak binding to the P- and C-domains was observed.

Identification of the region of C1q that binds to calreticulin

To identify the region of C1q that binds to calreticulin, a range of concentrations of whole C1q, the collagen-like tail region and the globular head region of C1q, as well as the recombinant globular head B chain region of C1q (U. Kishore, P. Eggleton and K. B. M. Reid, unpublished work), were immobilized on the surface of ELISA plates. A rabbit anti-(human C1q) polyclonal antibody was used to confirm that the C1q fragments had bound to the plates in a dose-dependent manner. Calreticulin bound most strongly, and in a dose-dependent manner, to whole C1q and the globular head region of C1q, in particular the globular head B chain region (Table 2). This binding was enhanced at half ionic strength. Calreticulin bound very weakly to the collagenous tail region of C1q. C1q plays an important role in the prevention of the formation of IC and in the opsonization and clearance of any IC formed. The globular head region of C1q is important for binding to the Fc region of immunoglobulins. These results strongly suggest that calreticulin binding to C1q may interfere with C1q-mediated functions involving the globular head region.

Inhibition of complement-dependent haemolysis by calreticulin

Activation of the classical pathway of complement is brought about by the binding of the globular head regions of C1q to the Fc portions of antibody IgM or IgG in the IC. In view of the interaction between calreticulin and the C1q globular head region, a C1q-dependent haemolytic assay was used to study the effect of calreticulin on complement activation. The assay of C1q-dependent haemolysis requires C1q to be added back to C1q-deficient serum in order to reconstitute the C1 complex. In the present study, the addition of C1q (15 µg/ml) back to C1q-deficient serum was sufficient to lyse 70–80% of EA. This concentration of C1q was then chosen as the standard in a series of studies to determine whether C1q-calreticulin binding results in inhibition of C1q-dependent haemolysis. Initially, various

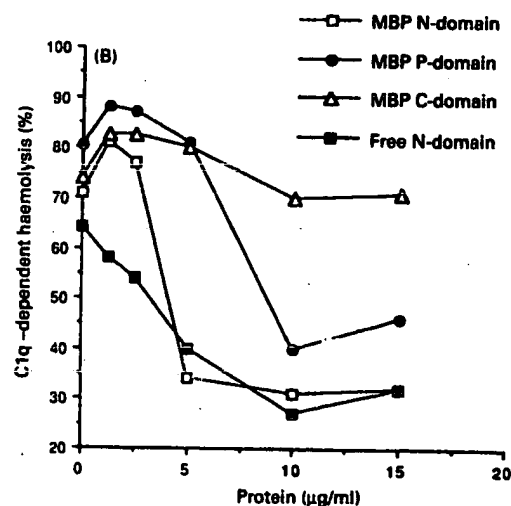
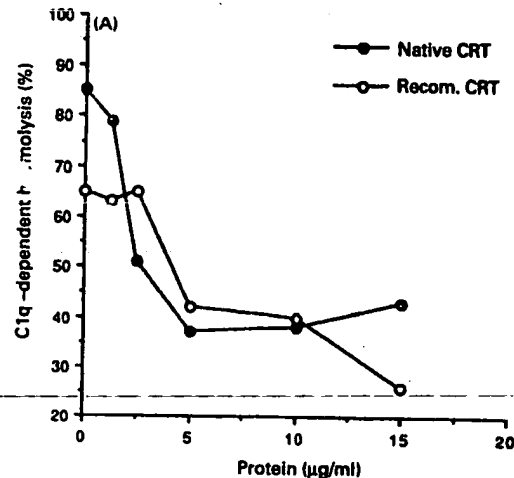


Figure 3 Inhibition by calreticulin of the C1q-dependent haemolysis of IgM-sensitized erythrocytes

(A) Different concentrations of native or recombinant (recom.) calreticulin (CRT) were added to a 1:40 dilution of C1q-deficient serum together with suboptimal amounts of C1q, and incubated for 1 h at 37 °C. Then 1×10^7 EA in 100 µl were added and incubated for an additional 1 h at 37 °C. The non-lysed cells were pelleted and the A_{450} values of the supernatants were measured. The percentage lysis was determined relative to complete (100%) lysis of cells. (B) Inhibition of the C1q-dependent lysis of EA by the N- and P-domains of calreticulin. Recombinant N-, P- and C-domains were employed in the assay described above. The means of three to six experiments are presented for each protein or domain.

concentrations of native calreticulin, as well as recombinant N-, P- and C-domains, at concentrations ranging from 0.125 to 40 µg/ml were incubated with 15 µg/ml C1q for 60 min at 37 °C before addition of the mixtures to EA. The addition of 2–5 µg/ml native, full-length recombinant calreticulin or N-domain lowered the haemolytic activity of C1q from 70–80% to below 20% (Figure 3A). A similar result was obtained when 10 µg/ml N- or P-domain was used instead of native calreticulin (Figure 3B). The C-domain of calreticulin had no effect on C1q-dependent haemolytic activity. Normal serum (diluted 1:20) and C1q-deficient serum containing no C1q or calreticulin were used as controls. The C1q-dependent haemolytic activity of normal

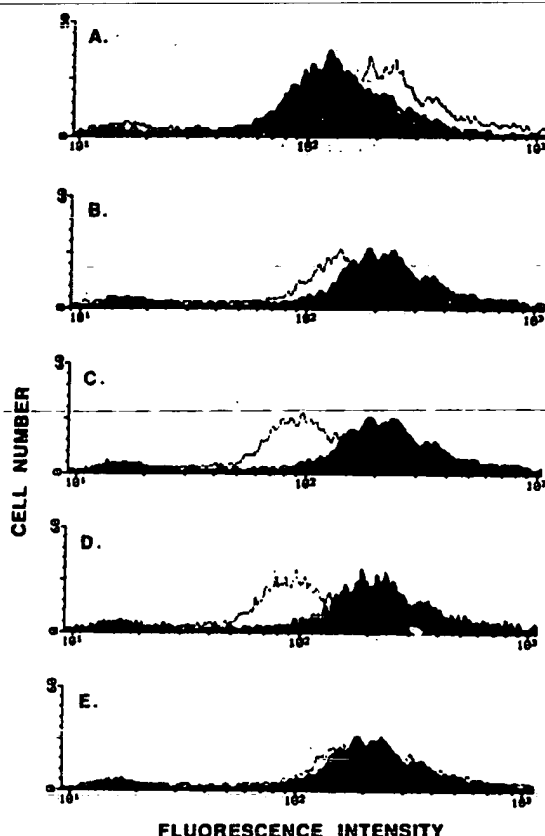


Figure 4 Flow cytometric analysis of the effects of C1q and calreticulin on binding of HAGG to neutrophils

C1q (30 μ g/ml) was incubated with calreticulin or one of its three functional domains, or with MBP alone, for 1 h at 37 °C. Then 50 μ g of HAGG was added followed by incubation with the various C1q-calreticulin preparations for a further 1 h at 37 °C, as described in the Materials and methods section. (A) HAGG binding alone (filled) or after incubation with C1q (open). (B)-(E) HAGG-C1q binding (filled) and decrease in HAGG-C1q binding in the presence of calreticulin (open): (B) full-length calreticulin, (C) N-domain, (D) P-domain, (E) C-domain. MBP alone had no effect on C1q-enhanced HAGG binding. The mean fluorescence intensities of control cells probed with primary antibody alone or with both primary and secondary antibodies were < 6 and < 60 respectively.

serum without the addition of calreticulin or additional C1q produced between 78 and 85% EA haemolysis, while C1q-deficient serum not supplemented with C1q or calreticulin resulted in only 18% haemolysis.

Effect of calreticulin on binding of HAGG to neutrophils

C1q is known to enhance IC binding and uptake by neutrophils [25]. In the present study, preincubation of 50 μ g of HAGG with 30 μ g of C1q led to an increase in HAGG binding to the cell surface (Figure 4A), increasing the mean fluorescence intensity from 158 to 293. As shown in Figures 4(B)-4(D), when preincubated with native calreticulin or its N- or P-domain, the C1q-mediated binding of HAGG to cells was impaired to various degrees, with the mean fluorescence intensity decreased from a value of 293 to values of 222, 197 and 132 respectively. The C1q-independent binding of HAGG via Fc receptors was not affected by preincubation with calreticulin or its domains. The C-domain of calreticulin (Figure 4E) did not impair the C1q-dependent

enhancement of HAGG binding to neutrophils. MBP was used as a control protein and had no effect on HAGG binding.

DISCUSSION

Calreticulin was first purified over 20 years ago [26], but the precise functions of this protein are still unknown. It has been implicated as an immunodominant antigen in autoimmune disease [3]. The protein has been localized to many intracellular and extracellular sites other than the ER, including the nuclear envelope [27], cytoplasmic granules [6], cell surface [8,28,29] and bloodstream [7,30]. The N-terminal amino acid sequence of one form of C1q receptor (C1qR; collectin receptor) is identical to that of calreticulin. In addition to binding to C1q, a C1q receptor has been proposed to be involved in the clearance of ICs from the circulation [31]. To date, it has been difficult to differentiate between C1qR and calreticulin at the biochemical and molecular levels. Further evidence that calreticulin and C1qR are similar proteins comes from our previous study in which C1q was found to bind to native full-length calreticulin [11]. In the present study, we wished to further dissect the molecular interaction between C1q and calreticulin and to address some of the biological implications of such an interaction. We observed the presence of detectable levels of calreticulin in the serum of approx. 20% of healthy individuals. We also demonstrated that stimulation of neutrophils, a major source of calreticulin, with FMLP can lead to the release of calreticulin *in vitro*. Calreticulin may be released from neutrophil minor granule stores. However, attempts to isolate calreticulin from the purified major primary and secondary granules of neutrophils have not been successful [11]. Alternatively, calreticulin may be released upon perturbation of the plasma membrane during cell activation. Stendahl and co-workers [32] have shown that calreticulin and the Ca^{2+} storage marker, a Ca^{2+} -dependent ATPase, both become concentrated in the filamentous actin-rich cytoplasmic area around ingested particles during neutrophil activation. This could be a means of calreticulin release by the cells during active phagocytosis of ICs or bacteria. Alternatively, FMLP is known to activate the rapid release of Ca^{2+} from intracellular stores and to induce degranulation of a number of secretory vesicles, which may account for the release of calreticulin into the extracellular environment. Moreover, recent evidence from Rosen and co-workers [33] concerning the morphological sequence of apoptosis has shown that many autoantigen clusters originating from the ER are contained within small blebs which are translocated to the cell surface. Calreticulin is believed to be present in these blebs, together with other autoantigens including Ro, La and nucleosomal DNA [33a]. The small particulate extracellular matter observed in the FMLP-treated neutrophils in the present study stained strongly for calreticulin, consistent with this mechanism of autoantigen translocation.

A functional interaction between calreticulin and proteins involved in vascular homeostasis has been examined previously. Calreticulin has been identified as an anti-thrombotic agent which binds to vitamin K-dependent coagulation factors, stimulates endothelial nitric oxide production and limits thrombosis in coronary arteries [30]. In order to study the interaction between calreticulin and the serum component C1q, the N-, P- and C-domains of calreticulin were expressed in *E. coli*. The first domain, which consists of the N-terminal half of the molecule, contains eight anti-parallel β -strands connected by protein loops. This domain is neutrally charged and has been found to contain binding sites for a number of proteins, including the DNA-binding domain of steroid receptors and the α -subunit of integrins [34,35]. The central P-domain comprises a proline-rich sequence

which, according to hypothetical modelling analysis, may keep the N- and C-terminal portions of the molecule spatially separate. The C-terminal region of the protein is highly acidic and negatively charged, and is thought to interact with blood clotting factors [30]. To test whether the binding of purified C1q to various domains of calreticulin is dependent upon ionic strength, some binding assays were performed under conditions of half-salt concentrations. Binding of C1q to the N-domain occurred under both physiological and half-salt conditions. However, binding of C1q to the P-domain only occurred under the more artificial low-ionic-strength conditions. The hypothesis that calreticulin-C1q complex-formation may be due to non-specific charge-charge interactions is unlikely, since the preponderance of negatively charged residues in the C-domain of calreticulin might have been expected to interact with charged regions of the C1q molecule; however, this is not the case. Having established the N- and P-domains of calreticulin as the sites for C1q interaction, we next examined which region of C1q binds to calreticulin. In ELISA, where native C1q, the collagen tail region, the native globular head region and the recombinant globular heads of C1q were immobilized, calreticulin bound most strongly to whole C1q and the globular heads of C1q, and insignificantly to the collagen tails.

Plasma C1q is the major molecule involved in the initiation of the complement cascade classical pathway. The globular head region of C1q binds specifically to ICs, initiating and subsequently amplifying the classical pathway of complement activation, and thereby preventing the formation of precipitating ICs in plasma. Thus the interaction of calreticulin with the globular heads of C1q probably interferes with mechanisms involving complement activation. In order to investigate this possibility further, a series of *in vitro* studies was undertaken. That calreticulin was in fact blocking the C1q-immunoglobulin interaction was confirmed when both native and recombinant full-length calreticulin were shown to be potent inhibitors of C1q-dependent haemolytic activity. The region involved could be localized to the N-terminal half (N- and P-domains) of the calreticulin molecule. Although C1q binds predominantly to the N-domain of calreticulin, which is also the most autoantigenic domain of the protein [21], incubation of C1q with the N-domain did not interfere with autoantibody binding to calreticulin (U. Kishore, K. B. M. Reid and P. Eggleton, unpublished work). This suggests that the main autoantigenic site on calreticulin and the C1q binding site are different.

The majority of circulating ICs are cleared by erythrocytes after binding to these cells via CR1 receptors. However, once ICs begin to accumulate in other tissues, leucocyte migration to these sites of IC deposition can play a role in their phagocytosis and clearance. IC binding to neutrophils is enhanced in the presence of 30 µg/ml C1q [25]. This suggests that binding of ICs to neutrophils not only is mediated through Fc receptors present on these cells, but is also enhanced by C1q-binding proteins on the cell surface, of which there are several candidates [28,36-38]. A 33 kDa C1q-binding protein, isolated from the plasma membranes of neutrophils [39], specifically binds to the globular head region of C1q and may function to enhance IC binding to neutrophils directly, or to enhance cross-linking of ICs to Fc receptors. Co-incubation of C1q with calreticulin partially prevented the enhanced binding of HAGG to neutrophils, mediated by C1q alone. These results suggest that calreticulin is capable of inhibiting the C1q-mediated binding of aggregated IgG to neutrophils, but not HAGG binding directly. Surprisingly, intact calreticulin appeared less effective than either the N- or the P-domain in inhibiting C1q-dependent binding of HAGG to cells. As the C-domain contains the majority of the acidic residues

found in the protein, the improved efficiency of the N- and P-domains in inhibiting the C1q-mediated binding of HAGG to cells may be due to elimination of the negative charge contained within the C-domain of the whole calreticulin molecule. That ionic interactions may be influential for the calreticulin-C1q interaction was suggested by our observations that the binding of C1q to the full-length calreticulin as well as to the N-domain increased with decreasing ionic strength. To date, there have been no reports that calreticulin binds specifically to immunoglobulins or interferes with immunoglobulin-mediated functions.

Confirmation of calreticulin release into the serum comes from two sources: first, the direct demonstration of elevated levels of calreticulin in the sera of SLE patients and second, the presence of autoantibodies against the protein in such sera. Calreticulin appears to interfere with the early phase of complement activation by (a) decreasing IC binding to leucocytes, and (b) inhibiting complement-dependent haemolysis. Such interactions with C1q could be of pathophysiological significance, whereby inhibition of complement activation may lead to a decrease in the efficient processing of ICs or the interactions of C1q with its various candidate receptors. Moreover, at sites in other tissues where ICs have accumulated, calreticulin may impede efficient IC binding and clearance by phagocytes recruited to the sites of inflammation. This is currently under investigation.

We thank Dr. T.-S. Lieu (University of Texas) for supplying the native calreticulin. This work was supported by Public Health Service Grants GM-37590 (K.S.Z.) and AI-33130 (K.N.S.), Applied Immune Sciences, Inc. (K.N.S.), the European Commission (U.K.) and the Medical Research Council (P.E., K.B.M.R.).

REFERENCES

- 1 Sontheimer, R. D., Maddison, P. J., Reichlin, M., Jorjani, R. E., Stasny, P. and Gilliam, J. N. (1982) *Ann. Intern. Med.* **97**, 664-671
- 2 Martinez-Lavin, M., Vaughan, J. H. and Tan, E. M. (1979) *Ann. Intern. Med.* **91**, 185-190
- 3 McCaulliffe, D. P., Lux, F. A., Lieu, T.-S., Sanz, I., Hanke, J., Newkirk, M. M., Bachinski, L. L., Itoh, Y., Siciliano, M. J., Reichlin, M., Sontheimer, R. D. and Capra, J. D. (1990) *J. Clin. Invest.* **85**, 1379-1391
- 4 Michalak, M., Milner, R. E., Burns, K. and Opas, M. (1992) *Biochem. J.* **285**, 681-692
- 5 Krause, K.-H., Simmerman, K. B., Jones, L. R. and Campbell, K. P. (1990) *Biochem. J.* **270**, 545-548
- 6 Dupuis, M., Schaerer, E., Krause, K.-H. and Tschopp, J. (1993) *J. Exp. Med.* **177**, 1-7
- 7 Sueyoshi, T., McMullen, B. A., Marnett, L. L., Du Clos, T. W. and Kisiel, W. (1991) *Thromb. Res.* **63**, 569-575
- 8 Zhu, J. and Newkirk, M. N. (1994) *Clin. Invest. Med.* **17**, 196-205
- 9 Unnasch, T., Gallin, M. Y., Soboslay, P. T., Ertmann, K. D. and Greene, B. M. (1988) *J. Clin. Invest.* **82**, 262-269
- 10 Khalife, J., Trottein, F., Schacht, A. M., Godin, C., Pierce, R. J. and Capron, A. (1993) *Mol. Biochem. Parasitol.* **57**, 193-202
- 11 Eggleton, P., Lieu, T. S., Zappi, E. G., Sastry, K. N., Coburn, J. P., Zaner, K. S., Sontheimer, R. D., Capra, J. D., Ghebrehewel, B. and Tauber, A. I. (1994) *Clin. Immunol. Immunopathol.* **72**, 405-409
- 12 Colomb, M. G., Arlund, G. J. and Villiers, C. L. (1984) *Complement* **1**, 69-80
- 13 Atkinson, J. P. (1989) *Clin. Exp. Rheumatol.* **7** (Suppl. 3), S95-S101
- 14 Schifferli, J. A., Bartolotti, S. R. and Peters, D. K. (1980) *Clin. Exp. Immunol.* **42**, 387-394
- 15 Zappi, E. G., Hasenauer, C. A., Lieu, T. S., Sontheimer, R. D. and Capra, J. D. (1991) *Clin. Res.* **39**, 496A
- 16 Eggleton, P., Wang, L., Penhallow, J., Crawford, N. and Brown, K. A. (1995) *Ann. Rheum. Dis.* **54**, 916-923
- 17 Reid, K. B. M. (1981) *Methods Enzymol.* **80**, 16-25
- 18 Paques, E. P. and Huber, R. P. H. (1979) *Hoppe-Seyler Z. Physiol. Chem.* **360**, 177-182
- 19 Reid, K. B. M. (1976) *Biochem. J.* **155**, 5-9
- 20 Kishore, U., Leigh, L. E. A., Willis, A. C., Eggleton, P. and Reid, K. B. M. (1996) *Mol. Immunol.* **33**, 88

21. Lieu, T., Newkirk, M., Capra, J. and Sontheimer, R. (1988) *J. Clin. Invest.* **82**, 96-101.
22. di-Guan, C., Li, P., Riggs, P. and Incuya, H. (1988) *Gene* **67**, 21-30.
23. Johnstone, A. and Thorpe, R. (1990) *Immunocytochemistry in Practice*, p. 50 Blackwell Scientific Publications, Oxford.
24. Kishore, U., Wang, J. Y., Hoppe, H.-J. and Reid, K. B. M. (1996) *Biochem. J.* **318**, 505-511.
25. Ohkuro, M., Kobayashi, K., Takahashi, K. and Nagasawa, S. (1994) *Immunology* **83**, 507-511.
26. Oswald, T. and MacLennan, D. (1974) *J. Biol. Chem.* **249**, 974-979.
27. Opas, M., Dziak, E., Fliegai, L. and Michalak, M. (1991) *J. Cell. Physiol.* **149**, 160-171.
28. Malhotra, R., Willis, A. C., Jensenius, J. C., Jackson, J. and Sim, R. B. (1993) *Immunology* **78**, 341-348.
29. Bijwaard, K., Hearing, V. J., Sontheimer, R., Lieu, T.-S., Vieira, W. D. and Gersten, D. M. (1995) *Melanoma Res.* **5**, 327-336.
30. Kuwabara, K., Pinsky, D., Schmidt, A., Benedict, C., Brett, J., Ogawa, S., Broekman, M., Marcus, A., Sciacca, R. and Michalak, M. (1995) *J. Biol. Chem.* **270**, 8179-8187.
31. Lozada, C., Levin, R., Huie, M., Hirschhorn, R., Nalme, D., Whitlow, M., Recl, P., Golden, B. and Cronstein, B. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8378-8382.
32. Stendahl, O., Krause, K.-H., Krischer, J., Jerstrom, P., Theiler, J., Clark, R., Carpentier, J. and Lew, D. (1994) *Science* **265**, 1439-1441.
33. Casciola-Rosen, L. A., Anhalt, G. and Rosen, A. (1994) *J. Exp. Med.* **179**, 1317-1330.
- 33a. Rosen, A., Casciola-Rosen, L. and Ahearn, J. (1995) *J. Exp. Med.* **181**, 1557-1561.
34. Burns, K., Duggan, B., Atkinson, E., Famulski, K., Nemer, M., Bleackley, R. and Michalak, M. (1994) *Nature (London)* **367**, 476-480.
35. Dedhar, S., Rennie, P., Shago, M., Hagesteln, C.-Y., Hawley, R., Bruchovsky, N., Cheng, H., Matusik, R. and Giguere, V. (1994) *Nature (London)* **367**, 480-483.
36. Guan, E. G., Burgess, W. H., Robinson, S. L., Goodman, E. B., McTigue, K. J. and Tenner, A. J. (1991) *J. Biol. Chem.* **266**, 20345-20355.
37. Eggleton, P., Ghebrehwet, B., Coburn, J. P., Sastry, K. N., Zaner, K. S. and Tauber, A. I. (1994) *Blood* **84**, 1640-1649.
38. Eggleton, P., Kishore, U., Leigh, L. E. A., Willis, A. C., Reid, K. B. M. and Ghebrehwet, B. (1996) *Mol. Immunol.* **33**, 88.
39. Eggleton, P., Ghebrehwet, B., Sastry, K. N., Coburn, J. P., Zaner, K. S., Reid, K. B. M. and Tauber, A. I. (1995) *J. Clin. Invest.* **95**, 1569-1578.

Received 24 July 1996/17 October 1996; accepted 7 November 1996

STIC-ILL

RC583.054
Adms
12.00

Fr m: Romeo, David
Sent: Monday, June 04, 2001 1:21 PM
To: STIC-ILL
Subject: 09503421 Kishore U; Sontheimer R D; Sastry K N; Zappi E G; Hughes G R; Khamashta M

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Kishore U; Sontheimer R D; Sastry K N; Zappi E G; Hughes G R; Khamashta M
A; Reid K B; Eggleton P
TI The systemic lupus erythematosus (SLE) disease
autoantigen-calreticulin can inhibit Clq association
with immune complexes.
SO CLINICAL AND EXPERIMENTAL IMMUNOLOGY, (1997 May) 108 (2) 181-90.
Journal code: DD7; 0057202. ISSN: 0009-9104.
AN 97301706

ADONIS - Electronic Journal Services

Requested by

Adonis

Article title	The systemic lupus erythematosus (SLE) disease autoantigen - Calreticulin can inhibit C1q association with immune complexes
Article identifier	0009910497005126
Authors	Kishore_U Sontheimer_R_D Sastry_K_N Zappi_E_G Hughes_G_R_V Khamashta_M_A Reid_K_B_M Eggleton_P
Journal title	Clinical and Experimental Immunology
ISSN	0009-9104
Publisher	Blackwell UK
Year of publication	1997
Volume	108
Issue	2
Supplement	0
Page range	181-190
Number of pages	10
User name	Adonis
Cost centre	Development
PCC	\$12.00
Date and time	Monday, June 04, 2001 5:13:30 PM

Copyright © 1991-1999 ADONIS and/or licensors.

The use of this system and its contents is restricted to the terms and conditions laid down in the Journal Delivery and User Agreement. Whilst the information contained on each CD-ROM has been obtained from sources believed to be reliable, no liability shall attach to ADONIS or the publisher in respect of any of its contents or in respect of any use of the system.

The systemic lupus erythematosus (SLE) disease autoantigen—calreticulin can inhibit C1q association with immune complexes

U. KISHORE, R. D. SONTHEIMER†, K. N. SASTRY*, E. G. ZAPPI†, G. R. V. HUGHES‡, M. A. KHAMASHTA‡, K. B. M. REID & P. EGGLETON *MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, Oxford, UK, *Department of Pathology, Boston University School of Medicine, Boston, MA and †Department of Dermatology and Internal Medicine, U.T. Southwestern Medical Center, Dallas, TX, USA and ‡The Rayne Institute, St Thomas' Hospital, London, UK*

(Accepted for publication 31 January 1997)

SUMMARY

Following its release from cells during infection and inflammation, calreticulin (CRT) can act as an autoantigen in diseases such as SLE. Why CRT is a target of protective immunity and whether it may interfere with innate immunity once released from cells during inflammation is unclear. In the present study, we found that CRT was detected more frequently in SLE sera and in higher amounts than found in control sera. Approximately 40% of SLE sera tested contained autoantibodies against CRT as detected by ELISA and immunoblotting. CRT was found to be predominantly in the sera of SLE patients associated with immune complexes and C1q; and only bound to the surfaces of neutrophils in the presence of low levels of calcium and magnesium. In order to further investigate the C1q–CRT interaction, recombinant CRT and its discrete domains (N-, P-, and C-domains) were produced in *Escherichia coli*. CRT binds to globular head region of C1q primarily via its N- and P-domains. The N-domain was shown to be the most autoantigenic region of CRT, as the anti-CRT autoantibodies from most patients reacted against this region. CRT also altered C1q-mediated immune functions. The P-domain of CRT bound to C1q and reduced the binding of immune complexes in SLE sera to immobilized C1q. Full length CRT and its N- and P-domains were able to reduce the C1q-dependent binding of immune complexes to neutrophils and solid-phase bound C1q. We conclude that CRT, once released from leucocytes during inflammation, may not only induce an antigenic reaction, but also interfere with C1q-mediated inflammatory processes.

Keywords calreticulin C1q immune complexes systemic lupus erythematosus

INTRODUCTION

A prototypical event in SLE is the formation and deposition of large quantities of antigen–antibody complexes (immune complexes (IC)) within various tissues of the body [1]. Once in the tissues, IC initiate inflammation through activation of the complement cascade pathway, which can ultimately lead to the tissue damage and disease manifestation. Complement activation normally helps prevent IC-mediated tissue injury by keeping IC small, rather than allowing them to form a range of insoluble lattices [2]. In SLE, it appears that both the prevention of the formation of large antigen–antibody complexes and subsequent clearance of immune complexes at the site of deposition have been impaired. Complement plays a role in the clearance and processing of IC in at least three ways: (i) by facilitating FcR-mediated phagocytosis of

IC; (ii) by inhibiting immune precipitation; and (iii) by mediating binding of IC to erythrocyte CR1. All these processes require activation of C1, the first component of the classical complement pathway. Initially the carboxy-terminal globular head region of the first subcomponent C1q binds to the CH2 domains of IC-fixed immunoglobulin [3]. C1q–IC are formed *in vivo* when the sub-components C1r and C1s become activated and initiate complement activity. The cascade is, in part, regulated by C1 inhibitor which binds to activated C1r and C1s, thereby inactivating these enzymes which become dissociated from the C1q–IC. In this process, complement is consumed during IC formation, which is reflected in patients with SLE who have decreased complement levels [4].

Recently, a 46-kD protein termed calreticulin (CRT), which runs aberrantly as a 60-kD protein on SDS–PAGE, has been proposed to be a new human rheumatic disease autoantigen [5]. Autoantibodies against CRT are found in many patients suffering from lupus disorders and Sjögren's syndrome [6,7]. CRT has been

Correspondence: Dr K. B. M. Reid, MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

isolated from many eukaryotic cell types with a concentration ranging from 20 to 500 µg/g of tissue and is particularly abundant in blood leucocytes. The precise function of CRT is still unknown, but the protein, by virtue of its KDEL carboxy-terminal sequence, is found associated with the lumen of the endoplasmic reticulum (ER), where it is believed to function as a high capacity Ca^{2+} storage and regulatory protein [8]. Several observations provide evidence that CRT can be the target of protective immunity. Homologues of CRT which show between 50% and 60% amino acid identity to human CRT have been found in the human parasites *Onchocerca volvulus* [9] and *Schistosoma mansoni* [10], which may help explain why CRT is a target for autoimmunity. CRT is normally intracellular, but has been found expressed on the surface of lung fibroblasts, with enhanced surface expression in response to cytomegalovirus infection [11]. Calreticulin is also a putative cell surface lectin which is required for B16 mouse melanoma cell spreading on laminin surfaces [12]. CRT has also been detected in low levels in the plasma of normal individuals [13] and it has been shown to bind to C1q [14].

It is now shown that higher concentrations of CRT levels were detected in SLE patient sera compared with control serum samples. IgG-specific autoantibodies to CRT were prevalent in 40% of SLE sera tested, and the antigenic domain of CRT is located towards the N-terminal region within amino acids 1–181. Examination of anti-CRT–CRT complexes showed that they were associated with C1q. Native and recombinant domains of CRT were used to determine if CRT–C1q interaction competitively inhibits the binding between C1q and endogenous immune complexes present in SLE sera. The N and P domains of CRT, when associated with C1q, appeared to reduce the ability of IC to bind to immobilized C1q and interfered with C1q-dependent IC binding to neutrophils. These results suggest that the release of CRT from leucocytes during inflammation may impede some of the functions of the classical complement pathway important for IC clearance.

PATIENTS AND METHODS

Patient sera

Sera from 24 patients whose diagnosis fulfilled four or more of the criteria of the American Association for Rheumatology [15] were used. Control sera were obtained from 24 healthy consenting adults.

Antibodies and other reagents

Affinity-purified rabbit antibodies raised against full length recombinant human CRT expressed in the baculovirus system [16] and against synthetic peptides (sp) corresponding to a sequence of residues located near the N-terminus of CRT (sp7–28) and the final 18 carboxy-terminal residues of human CRT (sp399–417), were prepared as previously described [17]. In addition, commercially prepared rabbit anti-human CRT was obtained from Affinity Bioreagents (Neshanic Station, NJ). Murine anti-human C1q was purchased from Quidel (San Diego, CA). Polyclonal anti-human C1q antisera were raised in rabbits by inoculating $3 \times 50 \mu\text{g}$ purified human C1q by intramuscular injection emulsified with 0.5 ml Freund's complete adjuvant in a total volume of 1 ml over 3 monthly intervals. The IgG fraction of C1q anti-serum was prepared by passing serum through a Pharmacia protein A column from a 4-month post-immunization bleed. FITC-labelled rabbit and mouse anti-human IgG were obtained from Jackson Immuno-Research Labs (West Grove, PA). Dulbecco's PBS with

and without calcium and magnesium was purchased from Sigma Chemical Co. (St Louis, MO). The expression vector (pMal-c2), amylose resin and Factor Xa protease were obtained from New England Biolabs (Beverly, MA).

Isolation and preparation of neutrophils

Human neutrophils were isolated from consenting normal donors by a one-step isolation procedure using polymorphprep (Nycomed, Birmingham, UK), as previously described [18]. The isolated cells (>95% pure) were then suspended in PBS-pH 7.4 without calcium or magnesium, and maintained at 4°C until use. The cells were then resuspended in the appropriate buffer. Isolated neutrophils were stained with trypan blue for 10 min and then examined by light microscopy to monitor the integrity of the plasma membrane of the cells before use.

Purification of native C1q and calreticulin

Haemolytically active C1q was isolated from human serum as described by Reid [19] and found to be pure as assessed by the A-B chains together (upper band of ~25 kD) and C chain alone (lower band of ~23 kD) characteristic banding, as seen on SDS-PAGE on a 12% (w/v) polyacrylamide gel under reducing conditions (Fig. 1, right panel). C1q was subjected to linear sucrose gradient centrifugation (5–31%), which confirmed the presence of a single non-aggregated protein. As a precaution, immediately before use, C1q was centrifuged at 14 000g for 15 min to eliminate any aggregates which may have formed during storage. Native CRT was purified employing the published method [20], and appeared on a 12% (w/v) SDS-PAGE gel as a 60-kD protein (Fig. 1, left panel). Albumin (67 kD) co-purified with CRT and appeared as the only contaminant.

Preparation of recombinant calreticulin and its N-, P-, and C-domains

For the expression and purification of recombinant proteins, a maltose binding protein (MBP) fusion system was used, consisting of pMal-c2 expression vector [21]. The details of recombinant proteins are being published elsewhere [22]. Briefly, the DNA sequences encoding for the full length CRT and its three functional domains [23] were polymerase chain reaction (PCR)-amplified using terminal primers and a 1.9 kb SacI cut cDNA encoding human CRT as template. Various regions of the protein were expressed, fused in-frame with the MBP and a Factor Xa protease site. The fusion proteins were purified on amylose resin columns, and cleaved to release the recombinant products with the expected N-termini (as checked by protein sequencing). For the purpose of this study, we expressed human CRT in three domains: N-terminal domain (N-domain), amino acids 1–180; proline-rich domain (P-domain), amino acids 181–290; and C-terminal domain (C-domain), amino acids 291–400, as well as full length CRT fusion protein of approximate molecular mass of 92 kD (Fig. 1, middle panel).

Dot blotting of sera for calreticulin

A BioRad dot-blot apparatus was used to prepare nitrocellulose filters (Hybond-C; 0.4 µ; Amersham, Aylesbury, UK) loaded with 1-µl aliquots of sera from 24 SLE patients and 12 control subjects, together with purified CRT. Nitrocellulose filters were then saturated with 2% w/v bovine serum albumin (BSA) PBS containing 0.05% v/v Tween 20 (PBS-T). The dot blotting analysis was performed using a 1:5000 dilution of rabbit polyclonal anti-human

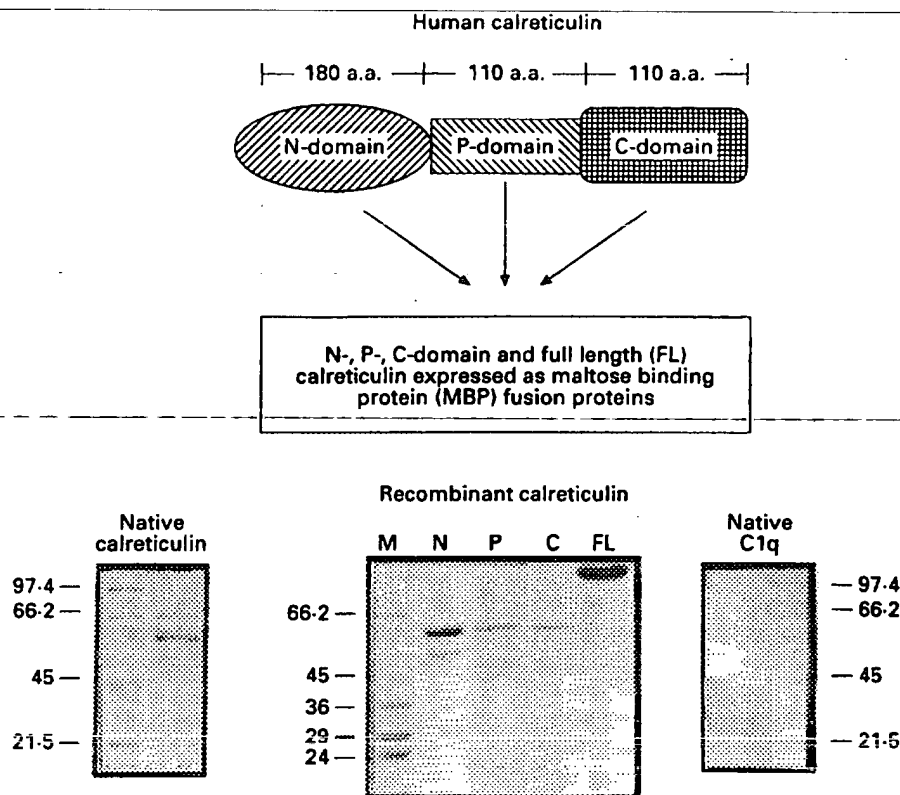


Fig. 1. Reduced SDS-PAGE (15%) of purified native (left box) and recombinant calreticulin maltose-binding fusion proteins (middle box) and native C1q (right box).

CRT (Affinity Bioreagents) raised against the full length molecule. After extensive washing in PBS-T, goat anti-rabbit IgG conjugated to peroxidase (Sigma) was used as a second antibody at the same dilution. The dot blots were developed using horseradish peroxidase luminescent visualization system for Western blotting (National Diagnostics, Atlanta, GA) and exposed to Fuji RX x-ray film for 12 min. Control filters were prepared in an identical fashion, except the primary antibody was replaced with 1:5000 of rabbit preimmune sera instead of anti-human CRT antisera.

Measurement of calreticulin and C1q levels in SLE and control sera by ELISA

For the detection of CRT and C1q levels in sera, whole blood was collected from 24 healthy control blood donors and 24 SLE patients and the serum was collected. Quaduplicate sera samples were diluted 1:100 in sodium carbonate buffer (0.015 mol/l Na_2CO_3 /0.035 mol/l NaHCO_3 , pH 9.6) and 100- μl volumes placed in Nunc Polysorb 96-well polystyrene plates. After incubation overnight at 4°C, wells were washed three times with PBS-T. Unoccupied absorption sites in the wells and non-specific protein-protein binding sites were blocked by 2 h incubation at 37°C with a 5% (w/v) powdered non-fat milk containing 0.1 mol/l glycine, and plates were again washed three times with PBS-T. Plates were then probed with either immunoaffinity purified rabbit anti-human CRT (1:4000 dilution), rabbit anti-human C1q or rabbit pre-immune sera for 1 h at 37°C. The use of pre-immune sera acted as a control, to assess non-specific binding of the antibodies and other proteins in rabbit serum that react with human C1q and CRT. Plates were washed three times with PBS-T, 100 μl of a peroxidase-conjugated goat IgG against human IgG (Sigma, Poole, UK)

diluted 1:1000 with PBS-T containing 0.2% w/v gelatin were added to each well, and after 1 h at 37°C, the wells were washed three times. Replicate wells containing sera samples were probed with second antibody alone to ensure non-specific antibody binding to the sera proteins was minimal. A volume of 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) in aqueous DMF mixed with 1/10th volume of hydrogen peroxide was added to each well. After incubation at room temperature for 20 min, the colour reaction was terminated by adding 150 μl of 2 N H_2SO_4 to each well and read at 450 nm. To assess the levels of CRT and C1q in sera, purity of CRT and C1q was assessed by SDS-PAGE (Fig. 1). Then standard curves were constructed using the same human CRT and C1q preparations, which were added back to CRT- and C1q-deficient serum over a concentration range of 1 $\mu\text{g}/\text{ml}$ to 0.02 $\mu\text{g}/\text{ml}$ in two-fold serial dilutions and detected by the ELISA procedure outlined above.

Capture ELISA for antibodies to calreticulin

A solid-phase indirect ELISA was used to detect calreticulin-binding autoantibodies in the serum samples from 24 SLE patients and 24 controls. Briefly, Nunc microtitre plate wells were coated overnight with 100 $\mu\text{l}/\text{well}$ of calreticulin or control antigens—BSA and mannan binding lectin (2.5 $\mu\text{g}/\text{ml}$) in sodium carbonate buffer pH 9.6. Unoccupied absorption sites were blocked by 2 h incubation at 37°C with a 5% w/v powdered milk/glycine blocking solution and then the plates were washed three times with PBS-T. Serum samples were diluted 1:100 in PBS-T pH 7.4 and 100- μl volumes were then added to the microtitre wells in duplicate and incubated at 37°C for 2 h. The wells were then washed and incubated with 100 μl of peroxidase-conjugated anti-human IgG

(1:5000; Sigma) for 1 h at 37°C. After washing three times, 100 µl of TMB (BioRad) were added to each well, plates were incubated for 20 min at 21°C and the enzyme reaction was terminated by addition of 150 µl of 2N H₂SO₄. Absorbance of wells was determined at 450 nm using a Titertec ELISA plate reader. Serum samples with a mean optical density (OD) value + 2 s.d. above the mean range of 24 normal sera were considered positive for anti-CRT antibodies. In order to identify further the region of calreticulin which was most antigenic, the N-, P- and C-domains of CRT fused to MBP, together with native CRT and MBP alone control proteins, all at 2.5 µg/ml sodium carbonate buffer, were coated in 100-µl volumes overnight at 4°C on ELISA plates. The ELISA as performed above was repeated.

Competitive inhibition ELISA assays were performed as described above with one exception. During the blocking buffer incubation step, human sera from SLE patients positive for anti-CRT antibodies were preincubated for 1 h at 37°C with concentrations of CRT ranging from 1 to 10 µg/ml (final concentration) before their addition to the solid-phase CRT or control antigen plates.

Western blot analysis of sera for anti-calreticulin antibodies

Immunoblot analysis was carried out to evaluate human CRT immunoreactivity and to confirm the positive and negative ELISA results. Briefly, purified human CRT was electrophoresed (5 µg/lane) under reducing conditions on a 15.0% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes at 0.8 mA/cm² over 90 min. Blots were blocked with 2.0% w/v BSA/PBS-T overnight at 4°C. The strips were then incubated with control sera or SLE sera positive or negative for anti-CRT antibodies (as determined by ELISA) for 3 h at 37°C. After washing, incubation with anti-human peroxidase-conjugated antisera (1:500 with 2% BSA/PBS-T) for 1 h at 37°C was performed. Development of the blot was achieved using horseradish peroxidase luminescent visualization system for Western blotting (National Diagnostics) and visualized on x-ray film (Fuji RX-film).

Detection of calreticulin-C1q complexes by capture ELISA

Nine SLE sera positive for antibodies against CRT were examined for C1q associated with the CRT. Rabbit affinity-purified anti-human CRT antisera (1:5000 dilution) were coated on duplicate wells overnight and subsequently blocked with 5% (w/v) non-fat milk. Aliquots of SLE sera (100 µl of 1:100 dilution) containing CRT were allowed to bind to the affinity-purified antisera for 2 h at 37°C, followed by extensive washing in PBS-T. To compensate for non-specific binding of immune complexes or C1q present in the diluted SLE sera to bound immunoglobulin on the plate or to the plate itself, replicate plates were coated with 1:5000 dilutions of pre-immune rabbit IgG. The captured complexes were then probed with 1:5000 dilution Fab₂ monoclonal anti-C1q for 1 h at 37°C, washed three times and then bound MoAb was detected with peroxidase-conjugated anti-mouse IgG and TMB as substrate. The OD reading of the paired control wells coated with pre-immune sera was subtracted from the test readings.

Binding of circulating IC from SLE sera and heat-aggregated human IgG to human C1q (100 ng/well) in the presence and absence of the P- and C-domain of CRT

Human C1q was coated on 96-well ELISA plates. Then either P-domain or C-domain of CRT (0.5 or 2.5 µg/ml) together with control proteins were allowed to bind to solid-phase C1q for 2 h at 37°C. Diluted test samples from six SLE sera and four control sera from laboratory personnel were added to the coated wells and

incubated for 2 h at 37°C to allow IC in the samples to bind to the C1q-coated wells. In addition, heat-aggregated human IgG (HAGG) at a similar concentration was also allowed to bind to replicate wells. Following washing to remove unbound material, antibodies to human IgG labelled with horseradish peroxidase conjugate was added (1:5000 dilution) and colour developed using TMB as substrate. In control assays, antibody alone was added to immobilized C1q to ensure that the antisera were not directly binding to C1q. The intensity of the absorbance at 450 nm above control levels was measured, which appeared proportional to the amount of IC bound to the C1q in the presence and absence of P- and C-domain of CRT. The control protein, MBP, and the C-domain of CRT did not inhibit IC in SLE sera from binding to C1q.

Flow cytometric analysis of HAGG binding to neutrophils in the presence of C1q and calreticulin

IgG was isolated from human serum as described previously [24]. Purified IgG (18–20 mg/ml) in water was heated to 63°C for 30 min. The immunoglobulin was centrifuged at 14 000 g for 20 min to remove insoluble precipitates. The HAGG was then adjusted to a concentration of 10 mg/ml and stored at 4°C. C1q (30 µg/ml) was incubated with and without CRT (5 µg) and its domains for 1 h at 37°C, and then incubated again with HAGG at a final concentration of 50 µg/ml for an additional 1 h at 37°C. Aliquots (100 µl) of various preparations were then incubated with 5 × 10⁵ neutrophils in PBS for 30 min at 4°C. After washing, binding of HAGG, HAGG-C1q and HAGG-C1q-CRT preparations of proteins to neutrophils was determined by incubating the cells with 1:50 dilution of rabbit anti-human IgG, followed by 1:50 dilution of goat anti-rabbit FITC conjugated antibody. Analysis was performed on 5000 cells by flow cytometry and the mean fluorescent intensity (MFI) presented. As only a small percentage of IC binding to neutrophils is C1q-dependent, a double labelling approach was preferred to FITC-HAGG direct labelling, in order to enhance the sensitivity of the assay to detect differences in HAGG binding.

To assess if CRT may also bind directly to the surface of neutrophils, a series of experiments was conducted. The effects of calcium/magnesium and temperature on CRT binding to the neutrophil cell surface were evaluated by incubating 5 µg CRT in 100 µl PBS with and without calcium and magnesium at 4°C and 37°C for 30 min. Similarly, increasing concentrations of CRT were mixed with 5 × 10⁵ neutrophils, then washed, and bound CRT was detected by incubation with rabbit anti-human CRT polyclonal antibody at 4°C for 30 min and analysed by flow cytometry employing Lysis II software version 1.1 (Becton Dickinson, Oxford, UK).

Statistical analysis

Statistical differences between the binding of IC to C1q in the presence and absence of CRT and its domains and binding of antisera against CRT to CRT in the presence and absence of C1q were determined using Student's paired *t*-tests. Statistical analysis of autoantibody and protein levels in control and SLE sera were performed using non-parametric methods of analysis. Results are expressed as median (range) values. Differences between groups were analysed by the Wilcoxon test (Statworks 1.2a) computer program.

RESULTS

Levels of calreticulin in SLE sera and detection of autoantibodies against CRT

A semiquantitative screen of 24 SLE sera and 12 control sera by dot blot analysis suggested that SLE sera had detectable amounts

Table 1. Serum levels of calreticulin (CRT) and C1q in control subjects and systemic lupus erythematosus (SLE) patients

Protein	Control subjects	SLE patients
C1q		
Mean C1q ($\mu\text{g/ml}$)	56.4 ± 14.9 ($n = 24$)	38.5 ± 13.3 ($n = 24$)
Range	25–80 $\mu\text{g/ml}$	8–60 $\mu\text{g/ml}$
No. of subjects with no detectable C1q	0	0
CRT		
Total no. subjects	$n = 24$	$n = 24$
Mean CRT for all subjects	0.42 $\mu\text{g/ml}$	4.44 $\mu\text{g/ml}$
Median CRT for all subjects	0.00 $\mu\text{g/ml}$	0.20 $\mu\text{g/ml}$
Range	0.0–4.0 $\mu\text{g/ml}$	0.0–18 $\mu\text{g/ml}$
No. of subjects with detectable CRT	5	12
Mean CRT	1.94 $\mu\text{g/ml}$	8.15 $\mu\text{g/ml}$
Median CRT	0.20 $\mu\text{g/ml}$	2.80 $\mu\text{g/ml}$

of CRT to a higher degree than 12 control sera (data not shown). In order to determine if CRT levels differed in the serum of healthy subjects and SLE patients, CRT was measured in 24 SLE sera and 24 control sera by quantitative ELISA. As shown in Table 1, the overall mean concentration CRT in 24 SLE sera was 4.44 $\mu\text{g/ml}$ compared with 0.42 $\mu\text{g/ml}$ for 24 control subjects. Twelve of the 24 SLE sera tested were positive for CRT with a median concentration of 2.8 $\mu\text{g/ml}$ (range 0.5–18 $\mu\text{g/ml}$), whilst only five control sera had detectable levels of CRT with a median of 0.2 $\mu\text{g/ml}$ (range 0.1–4.0 $\mu\text{g/ml}$). The C1q levels in both groups were also measured and the median values were found to be 40.5 $\mu\text{g/ml}$ (range 8–60 $\mu\text{g/ml}$) and 64.5 $\mu\text{g/ml}$ (range 25–80 $\mu\text{g/ml}$) in the SLE and control groups, respectively.

As higher concentrations of CRT were found in some SLE sera, we sought to determine the level of autoantigenic response against the protein and also investigate if it was associated with the first component of complement-C1q in the clinical sera. An ELISA plate was coated with 250 ng/well CRT. Sera from 24 SLE patients and 24 control subjects were screened for autoantibodies against these proteins. The median OD value for CRT autoantibodies in the SLE sera was 0.43 (range 0.20–1.79) compared with no detection of autoantibodies in control sera (Fig. 2a). Nine SLE patients (40% of the total number studied) which showed a significantly higher autoantibody titre for CRT than the highest value observed in the control group (Fig. 2a) were selected for further studies.

To confirm that the antibodies detected in the SLE sera which bound to solid-phase CRT were not simply the result of rheumatoid factors binding to the plate in a non-specific manner, a competitive ELISA was used in parallel experiments, where SLE sera were preincubated with free CRT (1 $\mu\text{g/ml}$) before addition to CRT-coated plates. The OD values of the SLE sera in the absence of free CRT were 1.41 ± 0.27 (mean \pm s.e.m.), but diminished to 0.68 ± 0.09 (Fig. 2b), confirming that the antibodies were indeed recognizing CRT specifically. The mean OD of control sera never exceeded 0.16 ± 0.06 . Further confirmation of anti-CRT–CRT interactions was achieved when two of the SLE sera shown by ELISA to be positive for autoantibodies against CRT were tested for reactivity with the purified 60-kD CRT protein band by Western blotting. Both anti-CRT-positive SLE sera reacted with native calreticulin (Fig. 3), which confirmed that antibodies present in some SLE sera specifically recognized CRT. A control serum

sample from a healthy subject, as well a number of SLE sera from patients negative for anti-CRT antibodies, did not react with CRT present on the immunoblot, even after prolonged development of the blot. To establish if autoimmune complexes in sera from SLE patients contain both CRT and C1q, a capture ELISA was employed. ELISA plates were coated with a 1:5000 dilution of anti-CRT antisera, and these were used to screen nine SLE patient sera positive for anti-CRT (diluted 1:100 with PBS) for IC containing CRT. To ensure that CRT:IC were binding specifically to anti-CRT antisera and not simply binding non-specifically to rheumatoid factors, polyclonal antisera to human MBL acted as a control capture immunoglobulin which was bound to a separate series of wells on the same ELISA plate, to assess non-specific

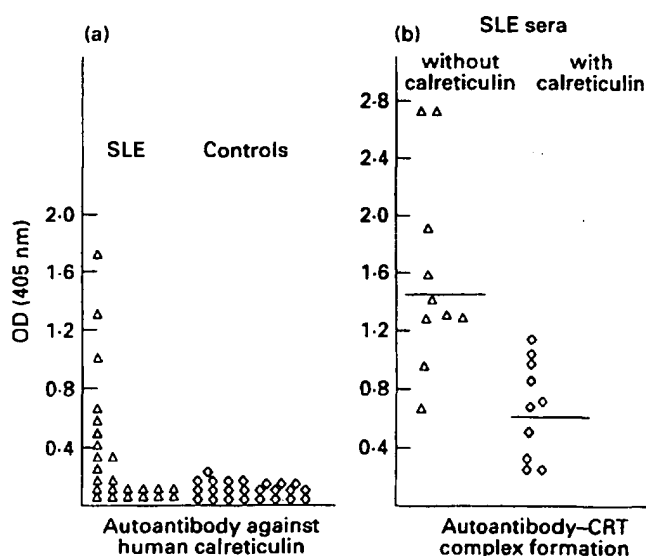


Fig. 2. Binding of sera from SLE patients and healthy subject to calreticulin (CRT) (a) and (b) competitive inhibition of anti-CRT antibodies in sera of patients with SLE selected from (a) from binding to immobilized CRT in the presence of free CRT. The presence of 100 ng free CRT inhibited binding of CRT-immune complexes to anti-calreticulin bound to the plates in the capture ELISA. Solid bars represent the mean optical density (OD) of each test group.

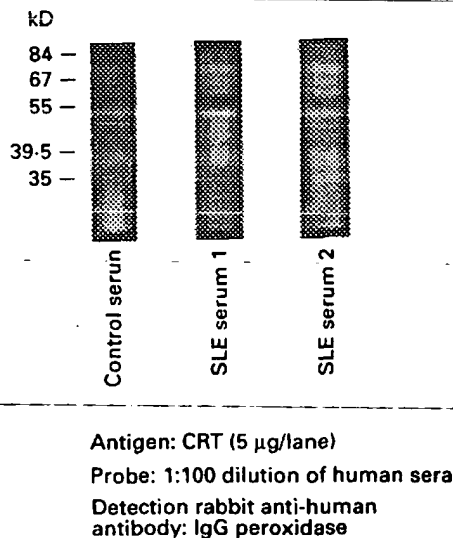


Fig. 3. Immunoblot analysis of control and SLE patient sera reactivity with human purified calreticulin (CRT) run under reducing conditions on a 15% SDS-PAGE gel and transferred to a PVDF membrane. Anti-human IgG-peroxidase antibody was used to detect binding of anti-CRT antibodies to calreticulin, as described in Patients and Methods.

binding of IC. The capture immune complexes were then probed with a 1:5000 dilution of monoclonal anti-C1q sera, which resulted in the detection of C1q associated with CRT:IC (Fig. 4).

Determination of the antigenic binding sites on CRT recognized by anti-CRT antibodies present in SLE sera

Sera from eight SLE patients identified as having anti-CRT antibodies were preincubated with fluid phase CRT over a concentration range of 1–10 µg/ml. As expected, CRT in the fluid phase competed for anti-CRT binding with the solid-phase bound material (Fig. 5a) in a dose-dependent manner. Having confirmed the presence of autoantibodies against CRT, we sought to define the domain of CRT which is recognized by the CRT autoantibodies in SLE sera. To localize the antigenic region of CRT, native and various recombinant domains were immobilized and screened for reactivity with SLE sera from patients known to contain anti-CRT antibodies. Control sera from laboratory personnel did not react with any of the domains of CRT tested. All the positive SLE sera reacted predominantly against the N-domain of CRT (Fig. 5b).

Effect of calreticulin on the ability of IC to bind to C1q

To investigate further if binding of CRT to C1q interfered with IC–C1q interaction, a series of 96-well plates was coated with C1q and/or maltose binding protein. The N and P domains of CRT were identified as the region of the protein which bound to whole C1q. However, the P- and C-domains were selected for this study to avoid complications in interpreting IC binding to C1q, or autoantibody binding to the N-domain of CRT, since the N-domain of CRT was also identified as the region of the protein that autoantibodies recognize. The P-domain was incubated with the immobilized C1q. Then, serum samples of SLE patients known to contain IC were added to the wells and allowed to bind for 1 h at 37°C. After washing, the ability of IC from six SLE sera to bind to C1q alone, or after binding to P- and C-domain of CRT, was assessed by ELISA. The results presented in Fig. 6a show that binding of 0.5 µg

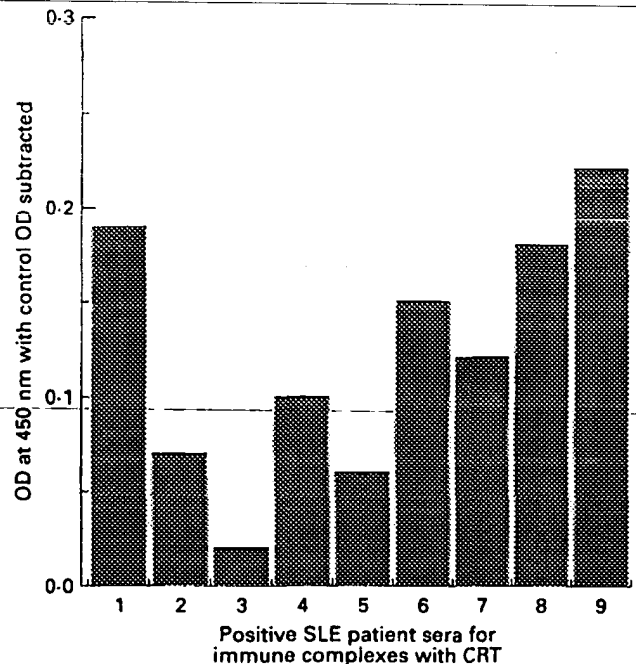


Fig. 4. Association of C1q with calreticulin (CRT) immune complexes in SLE sera as determined by capture ELISA. Diluted samples of rabbit anti-human anti-CRT antiserum (1:5000 in sodium carbonate buffer pH 9.6) were coated on ELISA plate wells overnight at 4°C. After washing, SLE sera positive for CRT were diluted 1:100 and added to the wells. After further washing, the presence of C1q was determined by probing with 1:5000 mouse anti-human C1q for 1 h at 37°C. The plates were then developed by ELISA and C1q was found to be associated with CRT-immune complexes in the selected SLE sera containing anti-CRT-immune complexes. A similar number of control sera were negative for C1q (optical density (OD) values <0.02) associated with CRT.

and 2.5 µg P-domain of CRT inhibited IC binding to C1q (Fig. 6a) by approximately 50–70% in six patient sera.

In order to confirm the apparent specificity of the C1q–P-domain interaction to interfere with IC binding to C1q, each domain was used at a range of concentrations to competitively inhibit HAGG binding to solid-phase bound C1q. Figure 6b shows the degree of binding by HAGG to C1q in the presence or absence of N-, P-, and C-domain of CRT. The N-domain at the highest concentration tested (2.5 µg/ml) inhibited binding of HAGG to C1q by approximately 50%, and the P-domain showed a similar degree of inhibition. The C-domain as well as MBP had no effect on HAGG binding to C1q. These data suggest that CRT and, in particular, the P-domain may bind to C1q and interfere with C1q–IC binding.

Effect of CRT on HAGG binding to neutrophils

C1q is known to enhance IC binding and uptake by neutrophils [24]. In this study, 50 µg of HAGG preincubated with 30 µg C1q led to an increase in HAGG binding to the cell surface (Table 2a) from an MFI of 158 to 293. As shown in Table 2a, when preincubated with native CRT or its N- or P-domain, the C1q-mediated binding of HAGG to cells was impaired to varying degrees, with MFI reducing from a value of 293 to values of 222, 197 and 132, respectively. The C1q-independent binding of HAGG via Fc receptors was not affected by preincubation with

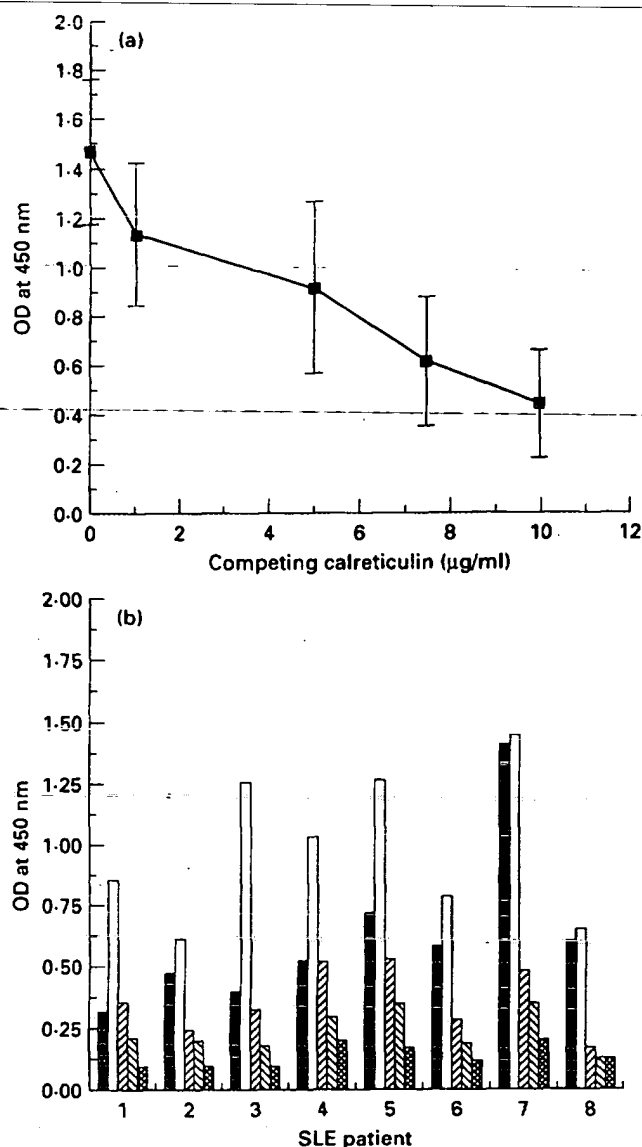


Fig. 5. (a) Competition by fluid phase calreticulin (CRT) for anti-CRT antibody binding to solid-phase CRT. Mean \pm s.d. binding activities of 100 μ l volumes of 1:50 dilutions of anti-CRT IgG antibodies from SLE sera to solid-phase bound CRT (100 μ l of a 1 mg/ml concentration), in the presence and absence of competing fluid-phase CRT over a concentration range of between 1 and 10 μ g/ml. The background of this assay was determined using a plate with wells coated with 100 μ l of 1 μ g/ml maltose binding protein (MBP) instead of CRT and was subtracted from all data. (b) Identification of the region of CRT which is recognized by SLE sera. Sera (1:100 dilution) from eight SLE patients were incubated for 1 h, 37°C with either immobilized native CRT or the N-, P- and C- MBP fusion domains of CRT. MBP fusion protein alone acted as a negative control. After incubation, individual wells were washed and probed with 100 μ l of 1:5000 dilution of anti-human IgG conjugated to peroxidase for 1 h, 37°C. Bound autoantibody was detected by ELISA. The optical density (OD) was read at 450 nm. ■, CRT; □, N-domain; ▨, P-domain; ▩, C-domain, MBP.

CRT or its domains. The C-domain of CRT did not impair C1q-dependent enhancement of HAGG binding to neutrophils. MBP, used as a control protein, had no effect on HAGG binding.

We wished to know if CRT could bind directly to neutrophils

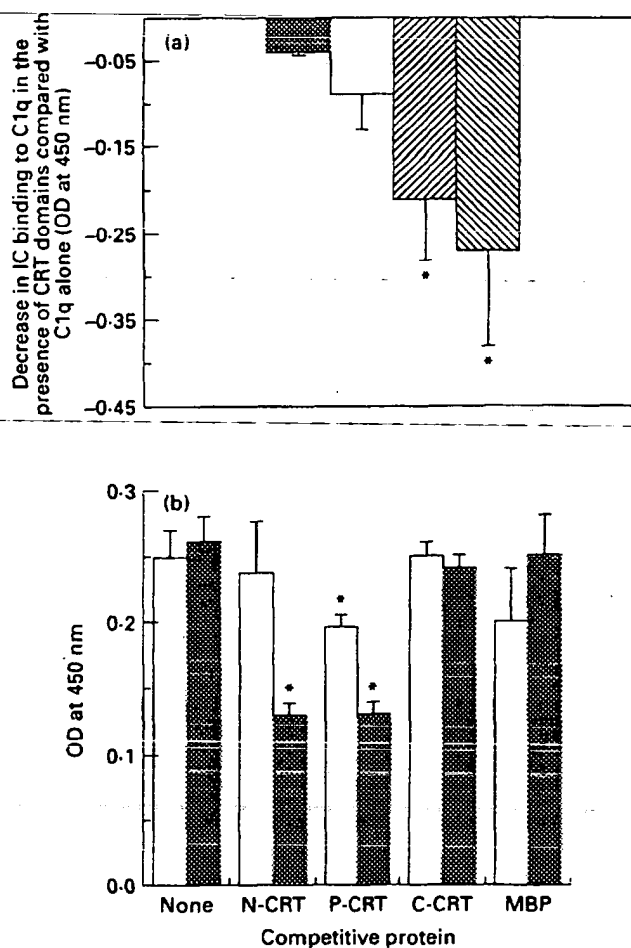


Fig. 6. Competitive inhibition, by the domains of calreticulin (CRT), with respect to: (a) percentage decrease in binding of immune complexes to C1q. The mean results \pm s.d. from six SLE sera are shown. ■, Maltose binding protein (2.5 μ g/ml); □, C-domain (2.5 μ g/ml); ▨, P-domain (0.5 μ g/ml); ▩, P-domain (2.5 μ g/ml). (b) Binding of heat-aggregated human IgG (HAGG) complexes to immobilized C1q after treatment with N-, P- and C-domain of CRT. Each point represents the mean \pm s.d. of triplicate determinations. * $P < 0.05$ compared with non-competitive protein control. □, 0.5 μ g/ml; ■, 2.5 μ g/ml. OD, Optical density.

under physiological conditions. Neutrophils (5×10^5 in total) were incubated with increasing amounts of native CRT at 37°C for 30 min. The intensity of CRT staining was assessed by flow cytometry. Binding of CRT to the neutrophil cell surface was enhanced in buffer without calcium and magnesium, as shown in Table 2b.

DISCUSSION

To date, the etiology of SLE remains unclear. However, the pathological characteristics of the disease are well documented and include the production of autoantibodies to some self antigens, formation of immune complexes, which can lead to acquired hypocomplementaemia and multi-organ immune-mediated damage. Recently, CRT has been described as a human rheumatic disease autoantigen [5]. In this study, we attempted to determine if CRT was detectable in SLE sera and assess the frequency and target site of autoantibodies against CRT in a number of randomly selected

Table 2. Effect of calreticulin (CRT) on C1q-mediated heat-aggregated human IgG (HAGG) binding to the cell surface and determination of the conditions for CRT binding to neutrophils

Neutrophil treatment	Mean fluorescence intensity (MFI)
<i>a. Effect of CRT on C1q-dependent HAGG binding to neutrophils</i>	
HAGG alone	158
HAGG + C1q	293
HAGG + C1q + CRT	222
HAGG + C1q + N-domain	197
HAGG + C1q + P-domain	132
HAGG + C1q + C-domain	282
<i>b. Conditions for CRT binding to neutrophils</i>	
Unstained	1.5
4°C, with Ca ²⁺ and Mg ²⁺	2.5
4°C, w/o Ca ²⁺ and Mg ²⁺	2.0
37°C, with Ca ²⁺ and Mg ²⁺	3.5
37°C, w/o Ca ²⁺ and Mg ²⁺	12.5

The effect of CRT on C1q-dependent HAGG binding to neutrophils was tested by incubating C1q (30 µg/ml) with CRT or its domains fused to maltose binding protein (MBP) for 1 h at 37°C. Then 50 µg of HAGG were incubated with the C1q-CRT domain preparations for another 1 h at 37°C. The MFI of control cells probed with primary antibody alone, or both primary and secondary antibodies, were <6 and <60, respectively. All data are representative results of at least two experiments. The conditions of CRT binding to neutrophils were determined by incubating the cells (5×10^5 assay) with 5 µg CRT for 30 min in PBS ± Ca²⁺ and Mg²⁺ at either 4°C or 37°C. Binding of CRT to the cell surface was detected by incubating with anti-human CRT, followed by staining with FITC-conjugated antisera, and analysed by flow cytometry.

SLE patients in comparison with healthy control subjects. Dot blot analysis suggested to us that CRT was present more frequently in SLE sera than control sera (data not shown). This observation was confirmed by quantitative ELISA. Relatively high concentrations of CRT were found in over 40% of sera from SLE patients, while either lower levels or no CRT could be detected in control sera. Approximately 50% of SLE sera tested contained autoantibodies against CRT. These results are consistent with earlier reports [25, 26]. In the patients studied here, there was not always a positive correlation between detectable CRT and the presence of anti-CRT antibodies in the same sera. Whether the CRT had been complexed with other autoimmune components and removed from the circulation is unknown and necessitates further investigation. Experiments were also carried out to locate the region of CRT which is a target for autoimmune response. Employing recombinant DNA techniques, the human CRT molecule was expressed as an N-terminal domain (amino acids 1–180); proline-rich P-domain (amino acids 181–290) and carboxy-terminal C-domain (amino acids 291–400). ELISA studies revealed that anti-CRT autoantibodies were directed against the N-terminal fragment. This supports the previous studies in which native Wil-2 cell CRT was proteolytically cleaved into a 23-kD N-terminal and 37-kD C-terminal fragment, of which the N-terminal fragment was found to be autoantigenic [27]. Synthetic peptides corresponding to amino acids 7–24 of human CRT have also been found to react specifically with anti-Ro/SS-A sera in ELISA [20]. A high degree of sequence homology exists between human CRT and the

causative agents of onchocerciasis (*O. volvulus*) and schistosomiasis (*S. mansoni*), which has led to the proposal that a cross-reactive immune response to CRT may occur [28]. However, amino acids 7–24 are not particularly conserved between human (KEQF-LDGDGWTSRWIE-S), *S. mansoni* (SETF-PNES-IENWVQ-S) and *O. volvulus* Ral-1 (KEDF-SDDDWEKRWIK-S) forms of CRT. It is therefore likely that different epitopes within the N-terminal region of CRT are targeted under various pathological conditions.

Of concern in this study was the observation that CRT interacts with the first subcomponent of complement, C1q. Interaction of CRT with serum proteins is not unprecedented, and CRT has been reported to bind to the vitamin K-dependent coagulation factor, Factor IX [29], which in turn binds to vascular endothelial cells. This may partially explain the divergent values of CRT seen in individual serum samples in this study. In a recent study in our laboratory [22], C1q was shown to bind predominantly to the N- and P-domains of CRT via its globular heads. However, preincubation of C1q with SLE sera containing anti-CRT autoantibodies did not interfere with the anti-CRT antibodies binding to immobilized CRT. This suggests that the autoantigenic site and the C1q binding site on CRT are distinct. Attempts to peptide map these two binding regions are in progress. Plasma C1q is the primary molecule for the initiation of the classical complement pathway. The globular head regions of C1q bind specifically to complexes of immunoglobulins, leading to efficient activation of the classical pathway of the complement system, and thus preventing the formation of precipitating IC *in vivo*. Impairment of

such mechanisms in SLE patients results in defective processing of IC [30,31]. To investigate further whether the interaction between CRT and C1q influenced the ability of C1q to bind to IC, a series of *in vitro* studies were undertaken. When C1q was immobilized, IC present in the SLE sera readily bound to C1q. However, in the presence of the P-domain of CRT, IC binding to C1q was reduced to varying degrees in six SLE patients. As CRT binds to the globular head of C1q, one would expect that CRT is preventing binding of the globular head region of C1q to the Fc portion of IC. The variation in impairment of IC binding to C1q by the P-domain may reflect the class or isotypes of antibodies making up the IC in individual patients. To ascertain further that both N- and P-domains of CRT can block binding of complexed IgG to solid-phase C1q without the complication of anti-C1q antibodies being present, as is the case in SLE sera, the study was repeated using heat-aggregated purified human IgG, where similar results were obtained. The evidence that CRT does, in fact, block C1q-immunoglobulin interaction was confirmed using a different approach in a parallel study, where both native and recombinant full length CRT were shown to be potent inhibitors of C1q haemolytic activity [29].

The majority of circulating immune complexes are cleared in the liver via erythrocytes after binding of the C3b-coated Fc to these cells via CR1 receptors. However, once IC begin to accumulate in other tissues, leucocyte migration to these sites of IC deposition can play a role in their phagocytosis and clearance. Immune complex binding to neutrophils is enhanced in the presence of 30 µg/ml C1q [32]. This suggests that binding of IC to neutrophils is mediated not only through Fc receptors present on these cells, but also by C1q binding proteins on the cell surface, of which there are several candidates [33–35]. A C1q binding protein of 33 kD (gC1qR) has been characterized and isolated from the plasma membranes of neutrophils [36], which specifically binds to the globular head region of C1q and may function to enhance IC binding to neutrophils directly, or cross-link IC to Fc receptors. Co-incubation of C1q with CRT partially prevented the enhanced binding of HAGG to neutrophils, mediated by C1q alone. These results suggest that CRT is capable of inhibiting C1q-mediated binding of aggregated IgG to neutrophils, but not HAGG binding directly to Fc receptors. To date, there have been no reports that CRT binds specifically to immunoglobulins or interferes with immunoglobulin-mediated functions.

In SLE sera, elevated levels of CRT and lower levels of C1q were found compared with normal sera. Confirmation of CRT release from leucocytes into the serum comes from two sources of data: first, direct measurement of CRT in serum of SLE patients, and second, the presence of autoantibodies against the protein in SLE sera. Possible pathological significance of CRT binding to C1q could be to partially impede IC binding to C1q and also decrease IC binding to leucocytes. These findings are similar to those found by Ahmed and co-workers [37], in which gp60, a normal plasma protein, inhibited C1q binding to IC and prevented complement-mediated prevention of immune complex precipitation. Therefore CRT together with other proteins such as gp60, IgM rheumatoid factor and other factors which can prevent the precipitation of IC, may impede efficient immune complex processing by complement and phagocytes recruited to the sites of inflammation.

ACKNOWLEDGMENTS

We thank Dr T.-S. Lieu (University of Texas) for supplying the native CRT.

This work was supported by the Arthritis and Rheumatism Council, the Medical Research Council of Great Britain (P.E., K.B.M.R.), the European Commission Biotechnology Programme (U.K.), Public Health Service Grant AI-33130 and Applied Immune Sciences, Inc. (K.N.S.).

REFERENCES

- 1 Cochrane CG, Koffler D. Immune complex disease in experimental animals and man. *Adv Immunol* 1973; **16**:185–253.
- 2 Schifferli JA, Bartolotti SR, Peters DK. Inhibition of immune precipitation by complement. *Clin Exp Immunol* 1980; **42**:387–94.
- 3 Colomb MG, Arlund GJ, Villiers CL. Structure and activation of C1: current concepts. *Complement* 1984; **1**:69–80.
- 4 Atkinson JP. Complement deficiency: predisposing factor to autoimmune syndromes. *Clin Exp Rheumatol* 1989; **7**(S-3):S95–S101.
- 5 Sontheimer RD, Lieu T-S, Capra JD. The diverse functional repertoire of calreticulin, a new human autoantigen. *The Immunologist* 1993; **1**:155–60.
- 6 Martinez-Lavin M, Vaughan JH, Tan EM. Auto-antibodies and the spectrum of Sjogren's syndrome. *Ann Intern Med* 1979; **91**:185–90.
- 7 Sontheimer RD, Maddison PJ, Reichlin M *et al.* Serologic and HLA associations in subacute cutaneous lupus erythematosus, a clinical subset of lupus erythematosus. *Ann Int Med* 1982; **97**:664–71.
- 8 Michalak M, Milner RE, Burns K *et al.* Calreticulin. *Biochem J* 1992; **285**:681–92.
- 9 Unnasch T, Gallin MY, Soboslay PT *et al.* Isolation and characterization of expression cDNA clones encoding antigens of *Onchocerca volvulus* infective larvae. *J Clin Invest* 1988; **82**:262–9.
- 10 Khalife J, Trottein F, Schacht AM *et al.* Cloning of the gene encoding a *Schistosoma mansoni* antigen homologous to human Ro/SS-A autoantigen. *Mol Biochem Parasitol* 1993; **57**:193–202.
- 11 Zhu J, Newkirk MN. Viral induction of the human autoantigen calreticulin. *Clin Invest Med* 1994; **17**:196–205.
- 12 White TK, Zhu Q, Tanzer ML. Cell surface calreticulin is a putative mannoside lectin which triggers mouse melanoma cell spreading. *J Biol Chem* 1995; **270**:15926–9.
- 13 Sueyoshi T, McMullen BA, Marnell LL *et al.* A new procedure for the separation of protein Z, prothombin fragment 1-2 and calreticulin from human plasma. *Thromb Res* 1991; **63**:569–75.
- 14 Eggleton P, Lieu T-S, Zappi EG *et al.* Calreticulin is released from activated neutrophils and binds to C1q and mannan binding protein. *Clin Immunol Immunopathol* 1994; **72**:405–9.
- 15 Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**:1271–7.
- 16 Zappi EG, Haseman CA, Lieu T-S *et al.* Expression of the human 46 kDa Ro/SS-A autoantigen (calreticulin) as a recombinant protein (Abstr). *Clin Res* 1991; **39**:496A.
- 17 McCauliffe DP, Lux FA, Lieu T-S *et al.* Molecular cloning, expression and chromosome 19 localization of a human Ro/SS-A autoantigen. *J Clin Invest* 1990; **85**:1379–91.
- 18 Eggleton P, Wang L, Penhallow J *et al.* Differences in oxidative response of subpopulations of neutrophils from healthy subjects and patients with rheumatoid arthritis. *Ann Rheum Dis* 1995; **54**:916–23.
- 19 Reid KBM. Preparation of human C1q, a subcomponent of the first component of the classical pathway of complement. *Methods Enzymol* 1981; **80**:16–25.
- 20 Lieu T-S, Newkirk M, Capra JD *et al.* Molecular characterization of human Ro/SS-A antigen: amino acid terminal sequence of the protein moiety of human Ro/SS-A antigen and immunological activity to a corresponding synthetic peptide. *J Clin Invest* 1988; **82**:96–101.
- 21 di-Guan C, Li P, Riggs P *et al.* Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose binding protein. *Gene* 1988; **67**:21–30.
- 22 Kishore U, Sontheimer RD, Sastry KN *et al.* Release of calreticulin from neutrophils may alter C1q-mediated immune functions. *Biochem J* 1997; **332**:543–50.

- 23 Baksh SM, Michalak M. Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains. *J Biol Chem* 1991; **266**:21458–65.
- 24 Ohkuro M, Kobayashi K, Takahashi K *et al.* Effect of C1q on the processing of immune complexes by human neutrophils. *Immunol* 1994; **83**:507–11.
- 25 Routsias JG, Tzioufas AG, Sakarellos-Daitsiotis M *et al.* Calreticulin synthetic peptide analogues: anti-peptide antibodies in autoimmune rheumatic diseases. *Clin Exp Immunol* 1993; **91**:437–41.
- 26 Hunter FA, Burger BD, Schrohenloher R *et al.* Autoantibodies to calreticulin in the sera of systemic lupus erythematosus. *Arthritis Rheum* 1991; **34**:S75.
- 27 Lux FA, McCauliffe DP, Buttner DW *et al.* Serological cross-reactivity between a human Ro/SS-A autoantigen (calreticulin) and the lambda Ral-1 antigen of *Onchocerca volvulus*. *J Clin Invest* 1992; **89**:1945–51.
- 28 Zhou Y, Dziak E, Unnasch TR *et al.* Major retinal cell components recognized by onchocerciasis are associated with the cell surface and nucleoli. *Invest Ophthalmol Vis Sci* 1994; **35**:1089–99.
- 29 Kuwabara K, Pinsky D, Schmidt A *et al.* Calreticulin, an antithrombotic agent which binds to vitamin K-dependent coagulation factors, stimulates endothelial nitric oxide production, and limits thrombosis in canine coronary arteries. *J Biol Chem* 1995; **270**:8179–87.
- 30 Davies K, Peters AM, Beynon HL *et al.* Immune complex processing in systemic lupus erythematosus—*in vivo* imaging and clearance studies. *J Clin Invest* 1992; **90**:2075–83.
- 31 Davies KA, Schifferli JA, Walport MJ. Complement deficiency and immune complex disease. *Spring Sem Immunopathol* 1994; **15**:397–416.
- 32 Bobak DA, Frank MM, Tenner AJ. C1q acts synergistically with phorbol dibutyrate to activate CRI-mediated phagocytosis by human mononuclear macrophages. *Eur J Immunol* 1988; **18**:2001–7.
- 33 Guan EG, Burgess WH, Robinson SL *et al.* Phagocytic cell molecules that bind to the collagen-like region of C1q: involvement in the C1q-mediated enhancement of phagocytosis. *J Biol Chem* 1991; **266**:20345–55.
- 34 Eggleton P, Ghebrehwet B, Coburn JP *et al.* Characterization of the human neutrophil C1q receptor and functional effects of free ligand on activated neutrophils. *Blood* 1994; **84**:1640–9.
- 35 Eggleton P, Kishore U, Leigh LEA *et al.* Identification of C1q-binding proteins associated with the plasma membrane of human neutrophils. *Mol Immunol* 1996; **33**:88.
- 36 Eggleton P, Ghebrehwet B, Sastry KN *et al.* Identification of a gC1q-binding protein (gC1qR) on the surface of human neutrophils: sub-cellular localization and binding properties in comparison with the cC1q-R. *J Clin Invest* 1995; **95**:1569–78.
- 37 Ahmed AEE, Veitch J, Whaley K. Mechanism of action of an inhibitor of complement-mediated prevention of immune precipitation. *Immunol* 1990; **70**:139–44.

STIC-ILL

mic
QP501.847

From: Romeo, David
Sent: Monday, June 04, 2001 11:48 AM
To: STIC-ILL
Subject: 09503421 Malhotra R, Laursen SB, Willis AC, Sim RB.

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE
Malhotra R, Laursen SB, Willis AC, Sim RB.

Localization of the receptor-binding site in the collectin family of proteins.

Biochem J. 1993 Jul 1;293 (Pt 1):15-9.

PMID: 8328957

RESEARCH COMMUNICATION

Localization of the receptor-binding site in the collectin family of proteins

Rajneesh MALHOTRA,*† Steen B. LAURSEN,† Antony C. WILLIS* and Robert D. SIM*

*Medical Research Council Immunochimistry Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K. and †Institute of Medical Microbiology, Bartholins Building, University of Aarhus, University Park, 8000 Aarhus, Denmark

Collectin receptor (C1q receptor) has been shown to bind human C1q, mannose-binding protein (MBP), lung surfactant protein A (SP-A) and bovine conglutinin. These ligands have a similar ultrastructure, each consisting of collagenous and globular domains, but do not show a high degree of sequence similarity. For C1q and SP-A, it has been shown that both bind to cell-surface-expressed receptor(s) via their collagenous regions and this is likely to be the case with the other ligands. Within the collagenous region, near the 'bend' region of the collagen triple helix in C1q, MBP and SP-A, a cluster of similar charged

residues is observed. This region has been suggested to be associated with receptor binding. A similar region of charge density occurs close to the N-terminus of conglutinin. In this paper we describe a truncated form of conglutinin, which has 55 amino acids missing from the N-terminus and does not bind to the collectin receptor. The results presented here strongly indicate that receptor–ligand interaction is mediated via the N-terminal region of conglutinin, consistent with the earlier proposal for the binding site.

INTRODUCTION

Collectins are a group of soluble proteins each of which has collagenous and non-collagenous domains, and may be complement associated and/or have lectin activity [1]. Collectins include lung surfactant protein A (SP-A), mannose-binding protein (MBP), C1q, lung surfactant protein D (SP-D) and conglutinin. All the molecules belonging to the collectin family consist of multiple polypeptide chains each made up of a short non-collagenous N-terminal segment, followed by a region of collagen-like sequence (characterized by the repeating triplet sequence Gly-Xaa-Yaa, where Yaa is often a hydroxylated amino acid). The C-terminal portion of each polypeptide is non-collagenous, and contains, in SP-A, MBP, conglutinin and SP-D, a structure known as a C-type lectin domain, which indicates that these proteins exhibit calcium ion-dependent binding to carbohydrates. In C1q, the C-terminal halves of the polypeptide sequences contain an immunoglobulin-binding domain. The subunits of the proteins are built up by association of three polypeptide chains, the collagenous regions of which intertwine to form a collagen triple helix. In SP-A, C1q, conglutinin and MBP, the collagen triple helix is bent in each subunit, due to an interruption in the repeating Gly-Xaa-Yaa sequences of the polypeptide chains. The non-collagenous C-terminal halves form a globular 'head'. Six such subunits, in SP-A, C1q or MBP, associate to form the characteristic 'bunch of tulips' structure seen in electron microscopy. In this structure, the globular heads form the 'flowers', and the collagen helices form the 'stalks' (Figure 1a).

At least four collectins (C1q, MBP, SP-A and conglutinin) bind to a single receptor (collectin receptor) [2]. Binding of SP-D to the collectin receptor has not been firmly established. The collectin receptor (also called C1q receptor) is a 56 kDa glycoprotein [3]. The N-terminal amino acid sequence of isolated collectin receptor and the sequence of peptides obtained by

V8/trypsin digestion show a high degree of similarity to the cDNA-derived amino acid sequence of a human protein reported as a component of RoSSA [4] or as calreticulin [5]. The comparison of immunological, composition, cellular localization and peptide-sequence data between purified collectin receptor and an isolated RoSSA component or the RoSSA cDNA sequence indicates that collectin receptor and RoSSA are similar, but not identical, molecules [6]. Collectin receptor has been isolated from a number of cell types, including human tonsil lymphocytes, the monocytic cell line U937 [3], endothelial cells and platelets [7] and the lung alveolar type-II cell line A549 [8]. Although the binding characteristics of collectin–collectin receptor interaction are well documented [2,9], the site of binding between collectin and collectin receptor has not been investigated. The ligands of the collectin receptor have similar quaternary structures, but are not highly homologous at the primary structure level. We therefore examined the known sequences of the ligands to select regions of similarity, which might be involved in the receptor binding. Experimental data on collectin–collectin receptor interaction was then obtained to confirm localization of the binding site.

MATERIALS AND METHODS

SDS/PAGE

SDS/PAGE was carried out as described by Laemmli [10]. Samples were prepared (reduced or alkylated) as described in [11]. Proteins were detected with Coomassie Blue staining.

Protein purification

C1q was isolated from human serum as described by Reid [12]. Collagen stalks from C1q were generated by pepsin digestion of C1q and purified by the method described by Reid [12]. Human collectin receptor was purified from human tonsil lymphocytes

Abbreviations used: MBP, mannose-binding protein; SP-A, lung surfactant protein A; SP-D, lung surfactant protein D; PEG 6000, poly(ethylene glycol) 6000; buffer A, 145 mM NaCl, 0.05% Tween 20, 2 mM CaCl₂; conglutinin (T), truncated form of conglutinin; conglutinin (N), normal form of conglutinin.

† To whom correspondence should be addressed.

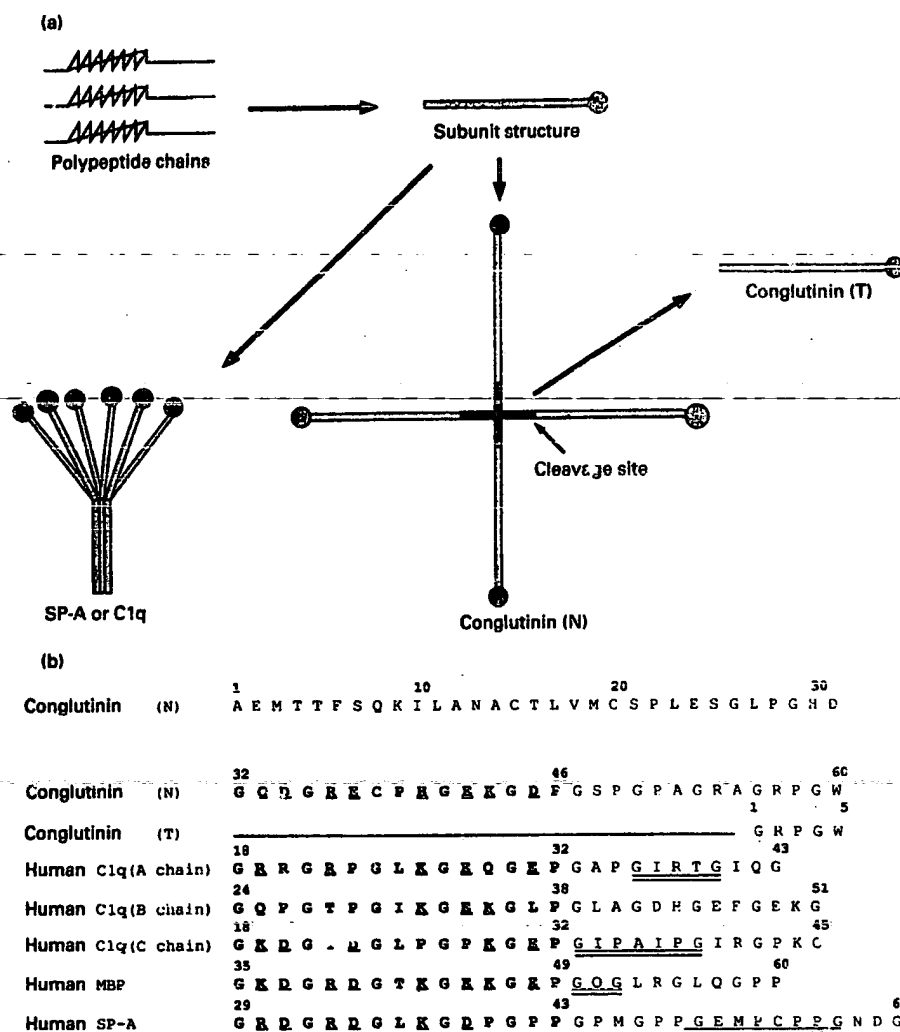


Figure 1 Schematic illustration of the assembly of SP-A, C1q and conglutinin (N) subunits from three polypeptide chains (a) and the N-terminal amino acid sequences (b) of the two forms of conglutinin, conglutinin (N) and conglutinin (T)

(a) Polypeptide chain contains a collagen-like region (represented by zigzag lines) and non-collagenous sequence (represented by straight horizontal lines). The ultrastructures of SP-A, C1q and conglutinin (N) are based on the appearance of these molecules in electron microscopy [22]. The likely mechanism by which conglutinin (T) is generated from the normal form and the cleavage site on conglutinin (N) are also shown. (b) The figure also shows the amino acid sequence of human C1q (A chain, B chain, C chain), human MBP and human SP-A in which there is an interruption to the Gly-Xaa-Yaa triplet are double underlined.

[3,6] and the purified receptor, as analysed by SDS/PAGE, is shown in Figure 2 (lane 1). Human SP-A was purified as described previously [1]. Bovine conglutinin was purified (S. B. Laursen, S. Thiel and J.-C. Janssens, unpublished work). In brief, conglutinin was precipitated from bovine serum with 3.5% (w/v) poly(ethylene glycol) 6000 (PEG 6000) (Sigma). The pellet was washed twice with 5 mM sodium barbitone buffer containing 145 mM NaCl, 0.05% Tween 20, 2 mM CaCl₂ (buffer A) and 3.5% (w/v) PEG 6000. The pellet was finally resuspended in buffer A and was loaded on to an *N*-acetyl-D-glucosamine-Sepharacryl S-300 column, pre-equilibrated with buffer A. *N*-Acetyl-D-glucosamine was coupled to Sepharacryl S-300 beads by the method of Fornstedt and P. Rath [13]. The column was washed extensively with buffer A and conglutinin-containing fractions were eluted with buffer A containing 2 mM *N*-acetyl-D-glucosamine. The conglutinin-containing fractions were dialysed

against buffer A and reloaded on to the *N*-acetyl-D-glucosamine-Sepharacryl S-300 column. Conglutinin-containing fractions were then eluted with buffer A containing 10 mM EDTA instead of 2 mM CaCl₂. These fractions were absorbed on a rabbit anti-(bovine immunoglobulin)-Sepharose column and then loaded on to a Sepharacryl S-400 gel-filtration column in buffer A. Two different forms of conglutinin were eluted from the gel-filtration column.

Radio-iodination of collectin receptor

Radio-iodination was carried out by the Iodogen method of Fraker and Speck [14]. The specific radioactivity of the labelled collectin receptor was 4×10^7 c.p.m./ μ g of protein, and the autoradiograph of the radiolabelled receptor, as analysed on SDS/PAGE, is shown in Figure 2 (lane 2).



Figure 2 SDS/PAGE analysis of unlabelled purified collectin receptor (lane 1) and autoradiograph of the dried SDS/PAGE gel of radiolabelled collectin receptor (lane 2)

Solid-phase binding assays

Microtitre plate wells were coated with 100 μ l of different forms of the purified congenitinin (50 μ g/ml) or BSA (50 μ g/ml) in PBS. Non-specific binding sites were blocked with 360 μ l of BSA (5 mg/ml) in PBS. Radio-iodinated collectin receptor (100 μ l; 2.3×10^5 c.p.m.) in 10 mM sodium phosphate buffer (pH 7.4) containing 0.1% Emulphogene was loaded on to the protein-coated wells and incubated for 1 h at room temperature. After extensive washing with loading buffer the bound radioactivity was eluted with 200 μ l of 4 M NaOH and the eluted radioactivity was measured.

In further sets of experiments microtitre plate wells were coated with C1q (10 μ g/ml) in PBS. Radio-iodinated collectin receptor (100 μ l; 2.3×10^5 c.p.m.) was incubated for 1 h at room temperature in the presence of serial dilutions of C1q collagen stalks (100 μ l; maximum amount 13 pmol), SP-A (100 μ l; maximum amount 4 pmol), the truncated form of congenitinin [congenitinin (T)] (100 μ l; maximum amount 63 pmol), the normal form of congenitinin [congenitinin (N)] (100 μ l; maximum amount 14 pmol), C1q (100 μ l; maximum amount 16 pmol) or BSA (100 μ l; maximum amount 63 pmol) in 10 mM sodium phosphate buffer containing 0.1% (w/v) Emulphogene (pH 7.4). The mixture was loaded on to the C1q-coated microtitre plate wells and incubated for 1 h at ambient temperature. After extensive washing with loading buffer the bound radioactivity was eluted with 200 μ l of 4 M NaOH and the eluted radioactivity was measured.

N-terminal amino acid sequence analysis

Samples for sequence analysis were run on SDS/PAGE under reducing conditions and electroblotted on to Problott membrane (Applied Biosystems, Warrington, U.K.) in a Bio-Rad mini

Trans-Blot electrophoretic transfer cell. The blots were stained with Coomassie Brilliant Blue and the bands corresponding to different forms of congenitinin were excised and sequenced using an Applied Biosystems 470A protein sequencer and Applied Biosystems 120A analyser.

RESULTS

In Figure 3(a) is shown SDS/PAGE analysis, under reducing conditions, of the purified congenitinin-containing fractions at different stages of purification. Two forms of the congenitinin polypeptide chain, molecular masses 40 kDa and 44 kDa, were co-purified from the *N*-acetyl-D-glucosamine-Sepharose S-300 column (Figure 3a; lane 4). These two species of congenitinin were separated by Sepharose S-400 gel filtration (Figure 3b) and the appearance of the two species of congenitinin polypeptide, after the gel-filtration step, is shown in Figure 3(a) (lanes 2 and 3). The elution profiles of the two forms of congenitinin, in comparison with standard proteins, by Sepharose S-400 gel filtration shows that the form containing the 44 kDa polypeptide chains, congenitinin (N), elutes between the void volume and the

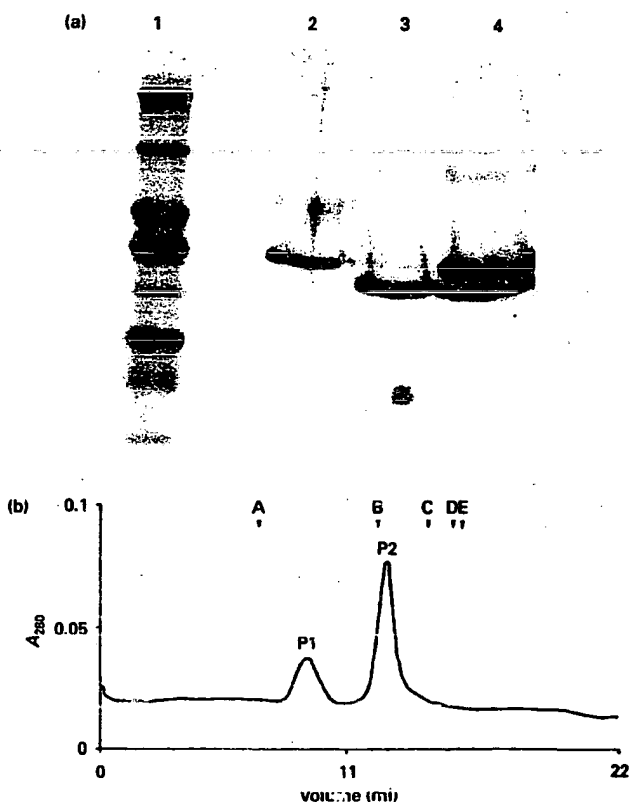


Figure 3 SDS/PAGE analysis of different forms of congenitinin (a) and elution positions of purified congenitinin (N) (P1) and congenitinin (T) (P2) from Sepharose S-400 gel filtration (b)

(a) Lane 1, molecular-mass standards: from top to bottom, myosin (212 kDa), phosphorylase b. (92 kDa), catalase (60 kDa), ovalbumin (42 kDa) and carboxypeptidase (25 kDa); lane 2, purified congenitinin (N) under reducing conditions (material from peak P1, Figure 2b); lane 3, purified congenitinin (T) under reducing conditions (material from peak P2, Figure 2b); and lane 4, fractions containing both forms of congenitinin after the *N*-acetyl-D-glucosamine-Sepharose S-300 column purification step, under reducing conditions. (b) Elution positions of molecular-mass markers: A, Blue Dextran (2000 kDa); B, thyroglobulin (669 kDa); C, ferritin (440 kDa); D, catalase (232 kDa); E, aldolase (158 kDa) are indicated by arrows.

670 kDa thyroglobulin marker. This is consistent with the very elongated shape of conglutinin. The form containing 40 kDa polypeptides, conglutinin (T), elutes at a slightly smaller size than thyroglobulin, indicating that it also is multimeric and elongated.

The N-terminal amino acid sequence analysis of these two forms of conglutinin revealed that the lower-molecular-mass species conglutinin (T) is a truncated form of the higher-molecular-mass species conglutinin (N) (Figure 1b). The origin of conglutinin (T) in serum is not clear, but it is likely to arise by proteolytic cleavage of conglutinin (N) (Figure 1a). Cleavage is at an Ala-Gly bond. Leucocyte elastase may be a candidate protease for cleavage, but there is no firm evidence of this as yet. The N-terminal sequence of conglutinin (T) (Figure 1b) indicates that 55 residues are missing from the N-terminus of each polypeptide chain compared with conglutinin (N). This missing portion, if it remained intact, could be in the form of four segments of triple helices, i.e. $4 \times 3 \times 55$ amino acid residues, or about 65–70 kDa. It may also, however, be further proteolysed to small fragments, or its polypeptides may dissociate. A thorough search of the fractions eluted from gel filtration (Figure 3b) failed to reveal any material corresponding to this other piece of conglutinin, or to sub-fragments of it. It is clear, therefore, that it dissociates from conglutinin (T) at an earlier stage (i.e. immediately after proteolysis, or during PEG 6000 precipitation, or during carbohydrate-binding affinity chromatography).

It has been shown by electron-microscopy studies that conglutinin (T), like conglutinin (N), forms the typical two-domain structure associated with collectins [15], i.e. both the forms have collagen-like stalks associated with globular heads. The major difference between the two forms of conglutinin is that conglutinin (T) is a single three-chain subunit, whereas conglutinin (N) forms a cross-like shape made up of four three-chain subunits (Figure 1a). As both molecules were eluted from the *N*-acetyl-D-glucosamine-Sephacryl S-300 column with EDTA and *N*-acetyl-D-glucosamine, it is clear that they retain lectin activity. This indicates that conglutinin (N) and conglutinin (T) both have a functionally intact C-terminal globular domain.

The binding of radio-iodinated collectin receptor to immobilized C1q is inhibited by both soluble C1q and soluble C1q-collagen stalks (Figure 4a), indicating that C1q interacts with collectin receptor through the collagen domain. This is consistent with the earlier reports that the binding of C1q and SP-A to cells expressing their receptor takes place through the collagen domain [16–19]. Consistent with earlier data [2], SP-A also inhibits C1q-collectin-receptor interaction (Figure 4a).

It has been shown previously that binding of SP-A and C1q to collectin receptor is dependent on ionic strength [1,3]. On the basis of binding characteristics and sequence comparison between the four collectins, i.e. MBP, SP-A, C1q and conglutinin, we had previously suggested that the region close to the collagen triple-helix bend region, a region of charged residues (Figure 1b), may be the binding site for SP-A or C1q with the collectin receptor [1]. Conglutinin (N) contains a similar region of sequence (Figure 1b), but this is absent from conglutinin (T). The use of these two forms of conglutinin, therefore, may provide evidence to support the suggested receptor-binding site.

Collectin receptor does bind to conglutinin (N) but not to conglutinin (T) (Table 1). Similarly the binding of collectin receptor to solid-phase immobilized C1q was inhibited by soluble C1q, and soluble conglutinin (N), but conglutinin (T) had only a minor effect on the interaction between collectin receptor and C1q (Figure 4b). The slight inhibition of binding of collectin receptor to C1q seen with higher concentrations of conglutinin (T) is probably due to minor contamination with conglutinin (N).

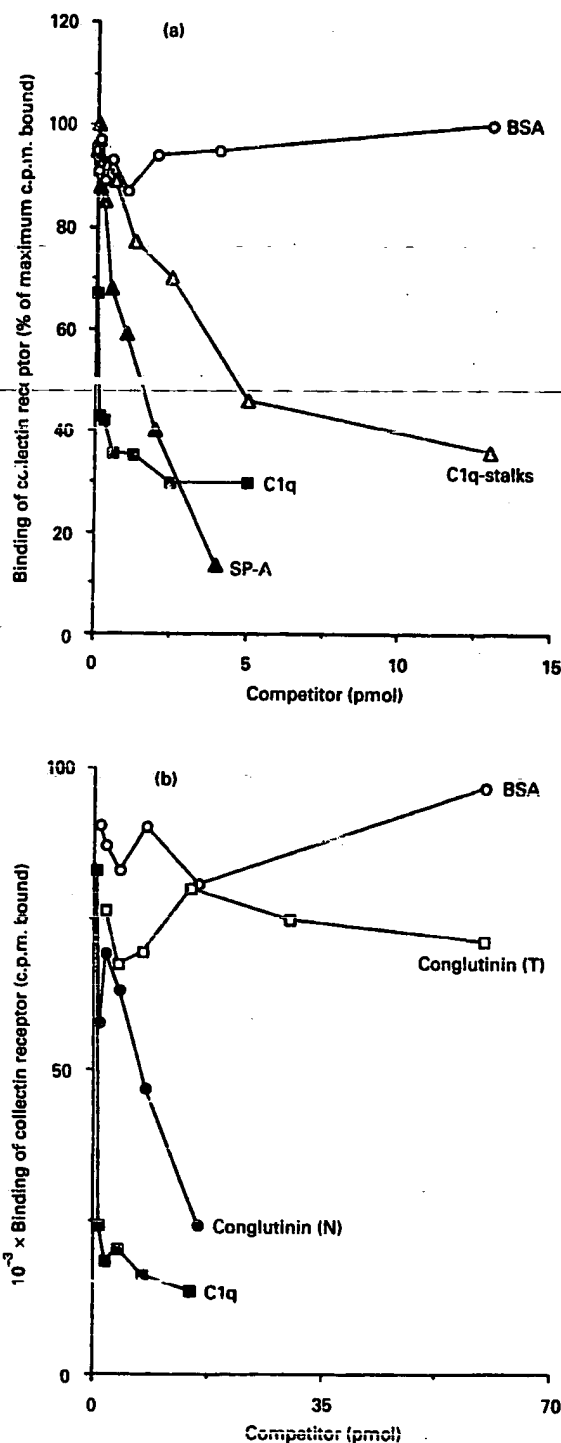


Figure 4 Binding characteristics of the collectin receptor

(a) Binding of radio-iodinated collectin receptor to solid-phase immobilized C1q in the presence of serial dilutions of C1q (■), C1q-collagen stalks (△), SP-A (▲) or BSA (○). Details are given in the Materials and methods section. Results are of three experiments, with the average of triplicate experimental points shown. (b) Binding of radio-iodinated collectin receptor to solid-phase immobilized C1q in the presence of serial dilutions of C1q (■), conglutinin (N) (●), conglutinin (T) (□) and BSA (○). Details are given in the Materials and methods section. Results are of three experiments, with the average of triplicate experimental points shown. To calculate the amount of collectins in terms of moles the molecular masses of collectins were assumed to be: C1q, 460 kDa; C1q-collagen stalks, 190 kDa; SP-A, 690 kDa; conglutinin (N), 528 kDa; conglutinin (T), 120 kDa.

Table 1 Binding of radiolabelled collectin receptor solid-phase immobilized conglutinin (N), conglutinin (T) and BSA

Details are given in the Materials and methods section. Results of two experiments each with the average of six experimental points and standard deviation are shown.

Ligand	Binding (c.p.m. bound \pm S.D.)
Conglutinin (N)	9700 \pm 713
Conglutinin (T)	979 \pm 284
BSA	562 \pm 121

(Figure 3a; lane 3). Conglutinin (T) differs from conglutinin (N) in that each polypeptide lacks the first 55 amino acids of the normal sequence (Figure 1b). The absence of this sequence clearly prevents interaction with receptor, as shown here, and also prevents formation of the four-subunit structure found in normal conglutinin [15]; Figure 1a).

DISCUSSION

We have previously shown that binding of SP-A and Clq to U937 cells and purified collectin receptor is dependent on ionic strength, implying involvement of charged amino acids in the interaction of collectins with collectin receptor [1,3]. The binding characteristics of interaction of SP-A with an SP-A receptor on human cell lines have not been extensively investigated, but a number of workers have characterized the binding of SP-A to rat alveolar type-II cells and rat alveolar macrophages. Rice et al. [18] and Wright et al. [20] provided evidence that the interaction of SP-A with receptor did not involve the lectin domain. Kuroki et al. [19] also showed that mannose or α -methylmannoside or concanavalin A did not effect the binding of radio-iodinated rat SP-A to alveolar type-II cells, nor did these substances affect the SP-A-induced inhibition of surfactant secretion. They also showed that chemical modification of basic amino acids in SP-A resulted in inhibition of binding of radio-iodinated SP-A to alveolar type-II cells and in inhibition of the biological activity of SP-A. This observation is consistent with the binding site suggested by us (Figure 1b). Interaction of dog SP-A with dog alveolar macrophages and polymorphs is destroyed by collagenase treatment [21]. In these earlier studies, the receptor to which SP-A was binding was uncharacterized, but it is now clear that SP-A binds to U937 cells and A549 cells via the collectin receptor [1,8]. The results presented in this paper show that the binding of collectin receptor to collectins takes place through the collagen domain and the binding site for collectin receptor on collectins consists of a region with a high proportion of charged

residues, which is consistent with the characteristics of SP-A or Clq interaction with different cell types as discussed above.

Binding of conglutinin (or other collectins) to the collectin receptor may be mediated by a linear sequence motif in the conglutinin polypeptide chain, or may require features of distribution of charge around a single collagen triple helix, or even around several helices, since in all the collectins the proposed binding site is in a region where several helices are in proximity. Whatever the requirement, the receptor-ligand interaction is clearly mediated via the N-terminal region of conglutinin, consistent with the earlier proposal for the binding site [1]. These results also indicate that collectin receptor is not a general receptor for collagen-like structures, but requires a specific charge distribution.

We thank Miss B. Moffatt and Mrs. J. U. Newell for technical assistance. We also thank Dr. K. B. M. Reid for useful discussion and advice. This project was funded by the British Lung Foundation and the Colt Foundation. R.M. is a British Lung Foundation Fellow.

REFERENCES

- Malhotra, R., Haurum, J., Thiel, S. and Sim, R. B. (1992) *Eur. J. Immunol.* **22**, 1437-1445
- Malhotra, R., Thiel, S., Reid, K. B. M. and Sim, R. B. (1990) *J. Exp. Med.* **172**, 955-959
- Malhotra, R. and Sim, R. B. (1989) *Biochem. J.* **218**, 625-631
- McCauliffe, D. P., Lux, F. A., Lieu, T.-S., Sanz, I., Hanka, J., Newkirk, M. M., Bachinski, L. L., Itoh, Y., Siciliano, M. J., Reichlin, M., Sontheimer, R. D. and Capra, J. D. (1990) *J. Clin. Invest.* **85**, 1379-1391
- Rokeach, L. A., Haselby, J. A., Meilof, J. F., Smeenk, R. J. T., Unnasch, T. R., Greene, B. M. and Hoch, S. O. (1991) *J. Immunol.* **147**, 3031-3039
- Malhotra, R., Willis, A. C., Jensenius, J. C., Jackson, J. and Sim, R. B. (1993) *Immunology* **78**, 341-349
- Peerschke, E. I. B., Malhotra, R., Ghebrehiwet, B., Reid, K. B. M., Willis, A. C. and Sim, R. B. (1993) *J. Leukocyte Biol.* **53**, 179-184
- Malhotra, R., Lu, J., Thiel, S., Jensenius, J.-C., Willis, A. C. and Sim, R. B. (1992) *Immunobiology* **184**, 437-438
- Pison, J., Wright, J. E. and Hawgood, S. (1992) *Am. J. Physiol.* **262**, L412-L416
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Farrar, G., Steck, T. L. and Wallach, D. F. H. (1971) *Biochemistry* **10**, 2602-2617
- Reid, K. B. M. (1981) *Methods Enzymol.* **80**, 16-25
- Fornstedt, N. and Porath, J. (1975) *FEBS Lett.* **57**, 187-190
- Fraker, P. J. and Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849-857
- Lu, J., Wiedemann, H., Holmskov, U., Thiel, S., Timpt, R. and Reid, K. B. M. (1993) *Behring Inst. Mitt.*, in the press
- Sobel, A. T. and Bokisch, V. A. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 965
- Arvieux, J., Reboul, A., Bensa, J. C. and Colomb, M. G. (1984) *Biochem. J.* **218**, 547-555
- Rice, W. R., Ross, G. F., Singleton, F. W., Dingle, S. and Whitsett, J. A. (1987) *J. Appl. Physiol.* **63**, 692-698
- Kuroki, Y., Mason, H. J. and Voelker, D. R. (1988) *J. Biol. Chem.* **263**, 17596-17602
- Wright, J. R., Wager, R. E. and Hawgood, S. (1987) *J. Biol. Chem.* **262**, 2888-2894
- Weber, H., Heilmann, P., Meyer, B. and Maier, K. L. (1990) *FEBS Lett.* **270**, 90-94
- Thiel, S. and Reid, K. B. M. (1989) *FEBS Lett.* **250**, 78-84

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/GB 98/02430

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/17 //C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 89 09273 A (BOARD OF REGENTS OF THE UNIVERSITY OF TEXAS SYSTEM) 5 October 1989 see the whole document ---	1-10
A	STUART G R ET AL: "Localisation of the C1q and collectin binding site within C1qR." 6TH EUROPEAN MEETING ON COMPLEMENT IN HUMAN DISEASE, INNSBRUCK, AUSTRIA, MARCH 12-15, 1997. EXPERIMENTAL AND CLINICAL IMMUNOGENETICS 14 (1). 1997. 37, XP002086303 see the whole document --- -/--	1-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

1 December 1998

Date of mailing of the international search report

14/12/1998

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

INTERNATIONAL SEARCH REPORT

Internatio. application No

PCT/GB-98/02430

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WEBSTER S ET AL: "Charge-based binding of complement component C1q to the Alzheimer amyloid beta-peptide." AMERICAN JOURNAL OF PATHOLOGY, (1997 MAY) 150 (5) 1531-6, XP002086304 see the whole document ---	1-10
A	STUART G R ET AL: "Localisation of the C1q binding site within C1q receptor/calreticulin." FEBS LETTERS, (1996 NOV 18) 397 (2-3) 245-9, XP002086305 cited in the application see the whole document ---	1-10
P,X	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US STUART G R ET AL: "The C1q and collectin binding site within C1q receptor (cell surface calreticulin)." XP002086306 see abstract & IMMUNOPHARMACOLOGY, (1997 DEC) 38 (1-2) 73-80, -----	1

Therapeutic uses of recombinant complement protein inhibitors

Kimberly R. Kalli¹, Peihong Hsu², and Douglas T. Fearon^{1,2,*}

¹ The Graduate Program in Immunology, and ² Division of Molecular and Clinical Rheumatology and Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Introduction

Complement acts in parallel and in concert with the immune system to protect the individual from microbial infection by mediating a variety of biologic reactions: increased vascular permeability, chemotaxis of leukocytes, opsonization, cytotoxicity of target organisms, and enhancement of the immune response. Activation of the complement system occurs via the alternative pathway in response to bacteria, yeast, virally infected cells [76, 84], or damaged tissue [10, 45], and via the classical pathway by particles or tissues recognized by classical pathway-activating antibodies [76, 84]. Both classical and alternative pathway activation serve to coat the offending surface with C3b and/or C4b by way of the metastable thioester in these proteins. C3b and C4b are structural subunits of the C3 and C5 convertases, which proteolytically cleave C3 and C5. When unregulated, the convertases amplify the initial deposition of C3b, and allow the cascade to proceed to the assembly of fragments C5b through C9, the membrane attack complex (MAC). Besides creating channels through the membrane which can cause cell death. Besides leading to cytolysis of activating cells, byproducts of C3 and C5 breakdown have additional inflammatory roles. The anaphylatoxins, C3a and C5a, induce histamine release by mast cells which leads to vasodilation and increased vascular permeability. C5a is a potent chemotactic factor which recruits and activates neutrophils, and both C3a and C5a induce rapid and enhanced production of leukotrienes from IL-3-primed basophils [11, 60]. Opsonization, or the deposition of C3b and C4b on an activating surface, marks the foreign particle with ligands for complement receptors involved in phagocytosis, and can lead to recognition by signaling receptors on phagocytes and lymphocytes specific for breakdown fragments of C3b.

While complement activation is a valuable first-line defense against potential pathogens, the activities of complement which promote a protective inflammatory

* Present address: Department of Medicine, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2SP, UK

Correspondence to: D. T. Fearon

K. A. Davies et al.

- Schifferli JA, Ng YC, Paccard J-P, Walport MJ (1989) The role of hypocomplementaemia and low erythrocyte complement receptor type 1 numbers in determining abnormal immune complex clearance in humans. *Clin Exp Immunol* 75: 329
- Schur PH, Marcus-Bagley D, Awdeh Z, Yunis EJ, Alper CA (1990) The effect of ethnicity on major histocompatibility complex complement allotypes and extended haplotypes in patients with systemic lupus erythematosus. *Arthritis Rheum* 33: 985
- Soeldner JS, Stone D (1986) Critical variables in the radioimmunoassay of serum insulin using the double antibody technique. *Diabetes* 14: 771
- Solal-Celigny P, Laviolette M, Herbert J, Atkins PC, Sirois M, Brun G, Lehner-Netsch G, Delage JM (1982) C3b inactivator deficiency with immune complex manifestations. *Clin Exp Immunol* 47: 197
- Späth PJ, Pascual M, Meyer-Häni L, Schaad UB, Schifferli JA (1988) Solubilization of immune precipitates by complement in the absence of properdin or factor D. *FEBS Lett* 234: 131
- Taylor RP, Wright EL, Pocianic F (1989) Quantitative analyses of C3b capture and immune adherence of IgM antibody/dsDNA immune complexes. *J Immunol* 143: 3626
- Taylor RP, Pocianic F, Reist C, Wright EL (1991) Complement-opsonized IgG antibody/dsDNA immune complexes bind to C3b clusters on isolated human erythrocytes. *Clin Immunol Immunopathol* 61: 143
- Tison RA, Winterborn MH (1981) Hypocomplementaemia due to a genetic deficiency of C3b convertase. *Clin Exp Immunol* 46: 110
- Uiger RD, Parker ML, Daughaday WH (1962) Studies on human growth hormone. I. A radioimmunoassay for human growth hormone. *J Clin Invest* 41: 254
- Valentin RM, van Overhagen H, Hazevoet HM, Hermans J, Cats A, Daha MR, van Es LA (1985) The value of complement and immune complex determinations in monitoring disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* 28: 904
- Van Dyne S, Holers VM, Lublin DM, Atkinson JP (1987) The polymorphism of the C4b/C4c receptor in the normal population and in patients with systemic lupus erythematosus. *Clin Exp Immunol* 68: 570
- Varga L, Thiry E, Fust G (1988) BSA-anti-BSA immune complexes formed in the presence of serum do not bind to autologous red cells. *Immunology* 64: 381
- Wallace JM, Wormald A (1931) Red cell adhesion in trypanosomiasis of man and other animals II. Some experiments on the mechanism of the reaction. *Parasitology* 23: 346
- Walport MJ, Peters AM, Elkon KB, Pusey C, Lavender JP, Hughes GRV (1985) The splenic extraction ratio of antibody-coated erythrocytes and its response to plasma exchange and pulse methylprednisolone. *Clin Exp Immunol* 60: 465
- Walport MJ, Ross GD, Mackworth-Young C, Watson JV, Hogg N, Lachmann PJ (1985) Family studies of erythrocyte complement receptor type 1 levels: reduced levels in patients with SLE are acquired, not inherited. *Clin Exp Immunol* 59: 547
- Walport MJ, Ng YC, Lachmann PJ (1987) Erythrocytes transfused into patients with SLE and hemolytic anemia lose complement receptor type 1 from their cell surface. *Clin Exp Immunol* 69: 501
- Wilson JG, Wong WW, Schur PH, Fearon DT (1982) Mode of inheritance of decreased C3b receptors on erythrocytes of patients with systemic lupus erythematosus. *N Engl J Med* 307: 981
- Wilson JG, Murphy EE, Wong WW, Klickstein LB, Weis JH, Fearon DT (1986) Identification of a restriction fragment length polymorphism by a CR1 cDNA that correlates with the number of CR1 on erythrocytes. *J Exp Med* 164: 50
- Wilson JG, Wong WW, Murphy EE, III, Schur PH, Fearon DT (1987) Deficiency of the C3b/C4b receptor (CR1) of erythrocytes in systemic lupus erythematosus: analysis of the stability of the defect and of a restriction fragment length polymorphism of the CR1 gene. *J Immunol* 138: 2708
- Wilson WA, Perez MC, Armatas PE (1988) Partial C4a deficiency is associated with susceptibility to systemic lupus erythematosus in black Americans. *Arthritis Rheum* 31: 1171
- Wong WW, Farrell SA (1991) Proposed structure of the FcγR1 allotype of human CR1. Loss of a C3 binding site may be associated with altered function. *J Immunol* 146: 656

ry reaction also carry the potential for harming the host by both indirect and direct mechanisms. For example, activated neutrophils are indiscriminate in their release of destructive enzymes, and complement components may be deposited on nearby cells as well as on targets. There is substantial evidence for the activation of the complement system in human disease, and for the dependency of tissue damage in animal models of disease on an intact complement pathway [18, 20, 23, 37, 61, 105].

Several proteins have evolved to control the extent of complement activation. The classical pathway C1 inhibitor will be discussed elsewhere in this volume, as will proteins which interfere with the insertion of the terminal components of the MAC, such as CD59 and homologous restriction factor. We will concentrate on proteins which have evolved to regulate complement activation at the level of C3 and C5 convertases, namely, members of the regulators of complement activation (RCA) gene family, and how they may be used to inhibit tissue damage due to complement in both antibody-independent and -dependent systems.

RCA family

The RCA gene family, summarized in Table 1 [14, 49], is a genetically linked group of proteins found in a 900-kb region on human chromosome 1, band q32 [12, 16, 66, 93, 95, 106]. Two of the RCA members, factor H and C4 binding protein (C4bp) are serum proteins, while decay-accelerating factor (DAF) [65], membrane cofactor protein (MCP) [62, 65], and complement receptors type 1 and

Table 1. Characteristics of the proteins of the regulators of complement activation

RCA proteins	Size (kDa)*	Ligands	Binding SCRs	Decay acceleration		Cofactor activity	
				C3bBb	C4b2a	C3b	C4b
Factor H	160	C3b	1-6 [4, 5]	+	-	+	-
C4bp	590	C4b	1-3 ^b [80]	-	+	-	+
DAF	70	C3b	2-4 [22]	+	+	-	-
		C4b	2-4				
MCP	58-63	C3b	2-4 [2]	-	-	+	+
		C4b	1-4				
CR1	190 ^c	C3b	8-11, 15-18	+	+	+	+
		C4b	1-4 [55, 57-59]				
CR2	140	C3dg	1-2 [15, 63]	-	-	^d	-
		iC3b	1-2 [54]				
		EBV	1-2 [15, 63]				

SCRs, Short consensus repeats; DAF, decay-accelerating factor; MCP, membrane cofactor protein; EBV, Epstein-Barr virus

* Apparent size on SDS-PAGE; of nonreduced samples

^b Murine C4bp

^c Most common allotype

^d iC3b

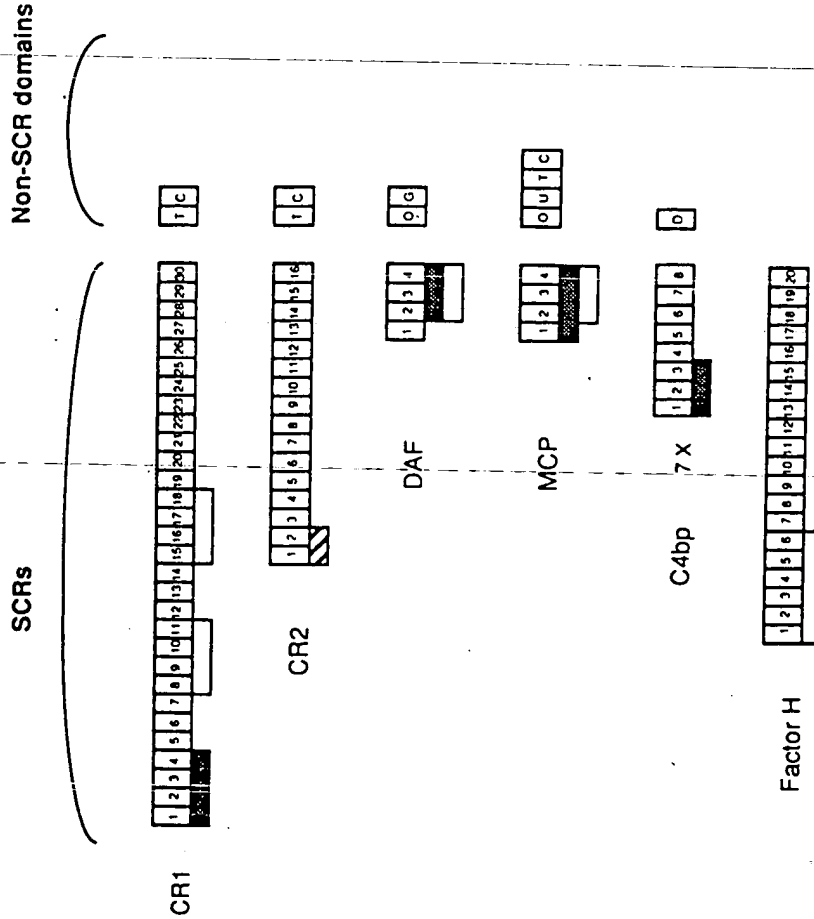


Fig. 1. Schematic representations of members of the RCA gene family, with closed boxes representing C4b-binding regions; open boxes, C3b-binding regions; cross-hatched boxes, iC3b-/C3dg-binding regions; O, site of O-linked glycosylation; G, glycolipid anchor; U, domain with unknown function; D, disulfide bridge-containing domain; T, transmembrane domain; and C, cytoplasmic domain

2 (CR1, CR2) [3] are membrane bound. They are structurally related proteins composed of 4 to 30 of the globular domains termed short consensus repeats (SCRs) [92] (Fig. 1). Each SCR is approximately 60 amino acids long, and has several conserved residues, including 4 cysteines which are disulfide linked within each SCR in a 1 to 3, 2 to 4 manner [53, 64]. Finally, the RCA proteins are functionally similar in that they recognize activation products of C3 or C4. Factor H [109] regulates alternative pathway complement activation in the fluid phase and on nonactivating surfaces, while C4bp inactivates classical pathway-activating particles containing C4b [35, 41, 98]. CR2, which has been reported to have weak cofactor activity for the proteolytic degradation of iC3b by the serum protease factor I [74], is more interesting for its signaling role on B lymphocytes [17, 43, 44, 68, 102].

DAF, MCP, and CR1 interact with C3b and C4b and regulate their ability to form C3 and C5 convertases. DAF accelerates the dissociation of the catalytic subunits of alternative and classical pathway C3 convertases, Bb, and C2a, re-

erisibly inactivates C3 or C5 convertases by acting as a cofactor for the proteolytic digestion of C3b or C4b. CR1 has all of these functions. It has been postulated that MCP and DAF have evolved cooperatively to protect autologous cells from complement-mediated damage, and that the function of CR1 is to inactivate C3b and C4b found on complement-activating complexes [6, 99].

The structure and function of DAF, MCP (and CR1) are discussed by Morgan and Meri elsewhere in this volume and a summary of their characteristics is shown in Table 1. However, CR1 is considered in detail here because it is the molecule which has principally been used for therapy.

CR1 (CD35). The most common allotype of CR1 is composed of 30 SCRs, 28 of which are arranged in tandemly repeating units of 7 SCRs termed long homologous repeats, or LHRs-A through -D, followed by two additional SCRs, a hydrophobic transmembrane domain, and a 43 amino acid cytoplasmic domain [57, 58, 48]. Deletion mutant analysis showed that the first two SCRs of LHR-A were responsible for C4b binding specificity, while the first two SCRs in LHRs-B are specified for C3b binding [58]. Thus, the most common allotype of CR1 is a total of three active binding sites, and is bivalent for C3b. Other more rare allotypes of CR1 [26–29, 113] are generally caused by the duplication or elimination of an intact LHR with C3b-binding function, leading to allotypes with more or fewer C3b-binding sites. This alteration can affect CR1 function, as assessed by the loss of affinity in binding C3b dimers, and a reduction in the effectiveness in inhibiting the C3 and C5 convertases [112]. The genomic structure of CR1 suggests that it evolved by duplication of segments of the gene encoding LHRs, perhaps by homologous recombination with unequal crossover [50, 114]. The extensive internal homologies between LHRs would facilitate their insertion or deletion to create the different alleles encoding the polymorphic allotypes of CR1, which differ by 30–40 kDa, the predicted size of an LHR. An additional CR1-like region [48, 50, 115] is found in the RCA locus, evidence of a non-functional rearrangement of the CR1 genetic elements.

Krych and colleagues [59] have mapped the amino acids in SCRs-8 and -9 which confer C3b-binding ability when transferred to the C4b-binding site in SCRs-1 and -2. Interestingly, these changes did not affect C4b binding. The limits of intact binding site were mapped with additional deletion mutants [55], indicating that the first four SCRs of either LHR-B (SCRs-8 through -11) or -C (SCRs-15 through -18) were necessary and sufficient to reconstitute the binding affinity for polymerized C3b and the cofactor activity of full-length CR1. As the third and fourth SCRs in LHR-A are virtually identical to those in the C3b-binding LHRs, it is postulated that these SCRs play a similar role in C4b binding. When DNA encoding SCRs-8 through -11 is inserted at the amino terminus of the secreted form of an immunoglobulin heavy chain gene and expressed in a cell with endogenous light chain, the purified chimeric protein retains all C3b-binding and cofactor functions of CR1, and has been shown *in vitro* to inhibit alternative pathway activation induced in human serum by zymosan [55].

CR1 is present on erythrocytes, B lymphocytes, neutrophils, monocytes, macrophages [31], and eosinophils, as well as on some T lymphocytes [111], follicular dendritic cells [94], glomerular podocytes [28, 56], Kupffer cells [46], and Schwann cells [116]. It was purified on the basis of its factor H-like ability to dissociate the alternative pathway C3 convertase and to act as a cofactor for the

factor I-mediated cleavage of C3b [30]. On a weight basis, it was eight-fold more active than factor H, and was not restricted by alternative pathway activating surfaces. Further studies showed that CR1 could act as a cofactor for C4b cleavage [51], thus inactivating the classical convertases as well as alternative pathway convertases. CR1 also accelerates the decay of each convertase [40, 51]. Unlike cells expressing MCP or DAF, CR1 expression on a cell allows it to rosette with C3b-bearing particles, or to bind soluble polymerized C3b, and to act as a cofactor for the cleavage of C3b and C4b in these particles [69, 70, 97]. Although CR1 has intrinsic activity as well as extrinsic activity [70], it is reasonable to postulate that the 90-nm extension of CR1 from the cell membrane [107] allows control of complement activation on adjacent complexes. CR1 has been implicated in controlling the solubility and clearance of immune complexes [21], participating in endocytosis of complexes and particles by neutrophils and macrophages [1], as well as having other putative immunoregulatory roles [3, 24, 108].

Potential clinical applications of RCA proteins

Complement has been shown to play a role in the pathogenesis of some types of inflammatory tissue damage initiated by both non-immunologic [39, 45] and immunologic [37, 61, 91, 105] conditions. In animal models, removal of classical pathway-activating antibodies [33, 87], depletion of complement by cobra venom factor [19, 32, 39, 61], or testing the models in animals genetically deficient in specific complement components [39, 105] abrogates or delays pathogenesis. Tissue injury can be caused by complement either directly or indirectly. The formation of the MAC on diseased or healthy cells may lead to cell lysis or even to activation, as for example occurs with endothelial cells in which MAC insertion results in calcium flux and expression of binding sites for various thrombolytic mediators [42], as well as release of heparin sulfate from endothelial surfaces to contribute to a hypercoagulable state [86]. Indirectly, the byproducts of C3 and C5 degradation recruit and activate neutrophils, and may synergize with interleukins to cause synthesis of inflammatory mediators such as leukotrienes [11, 60]. Neutrophil activation caused by the complement system has been implicated in damage to organs distinct from those in which activation has occurred, such as in adult respiratory distress syndrome (ARDS) [100].

For a complement inhibitor to be clinically useful, several requirements must be met. First, the molecule should be able to inhibit complement activation at the level of the C5 convertases of both alternative and classical pathways, not only to protect a cell from lysis, as may be achieved by CD59, but also to block the generation of C5a which would induce neutrophil influx and attendant inflammation. Secondly, an inhibitor should have a high affinity for the multivalent complexes of C3b and C4b found in the convertases. Third, it should irreversibly inactivate the convertases, and fourth, the inhibitor should be able to recycle and inhibit multiple convertases.

The members of the RCA family represent potential therapeutic inhibitors that can be evaluated according to these requirements. C4bp and factor H cause irreversible inactivation of C4b and C3b, but they are unlikely to be useful. The former acts only to prevent spontaneous, but not immune complex-induced classical pathway activation. As its major ligand is C4b, damage caused by primarily

alternative pathway activation or that recruited by classical pathway activation would be unaffected. The latter serves only to prevent spontaneous but not induced activation of the alternative pathway.

Except for DAF found in the fluid phase, which functions similarly to C4bp in C4b2a decay acceleration [72], DAF and MCP are intrinsic regulators of complement, which function only when incorporated into a cell membrane. DAF does not induce irreversible inactivation of C3b and C4b, is restricted by alternative pathway activating surfaces [83], and is not associated with a clinical phenotype in its absence on Inab blood group erythrocytes [47, 73, 101], all of which suggests a modest inhibitory potential. MCP meets several requirements by acting in both pathways, by irreversibly inhibiting the function of C3b and C4b, and by being able to recycle with additional substrate. These characteristics may indicate that MCP is a suitable candidate for a cell-bound inhibitor, but its monovalency and relatively low affinity suggest that it could not serve as an effective soluble inhibitor.

CR1 meets these requirements by being capable of inhibiting both the classical and alternative pathway C3 and C5 convertases, by interacting multivalently with these complexes through its three binding sites, by irreversibly inactivating C3b and C4b through promoting their proteolysis by factor I, and by recycling for inhibition of additional C3/C5 convertases after release from degraded C3b and C4b.

Applications of soluble CR1

A soluble form of CR1 (sCR1) was made by genetically engineering a stop codon in CR1 cDNA before the transmembrane and cytoplasmic domains [107]. When purified from the media of cells transfected with the altered cDNA, this molecule was able to bind C3b and serve as a cofactor for C3b cleavage. The classical pathway-mediated lysis of sensitized sheep erythrocytes and the alternative pathway activation induced by zymosan were efficiently inhibited by sCR1 at concentrations 100-fold lower than normal serum concentrations of factor H and C4bp [107]. This molecule has been tested for its ability to inhibit damage caused by complement in several models of disease (Table 2).

Non-antibody-dependent complement activation

Reperfusion injury. The complement system was suggested to have a deleterious effect on ischemic myocardium by the finding that administration of cobra venom factor to dogs prior to coronary artery occlusion decreased the size of the myocardial infarction [67]. The maintenance of viable myocardium was correlated to reduced neutrophil infiltration. To determine whether complement activation contributed to reperfusion injury of ischemic myocardium, 1 mg sCR1 was administered as a single bolus injection to rats immediately before temporary occlusion of the left coronary artery for 35 min, after which perfusion was reestablished. Seven days later, the animals were killed and sCR1 was found to have reduced the sizes of the infarcts by 44%. In a second group of rats subjected to myocardial ischemia and reperfusion, the mechanism of the protective effect of sCR1 was shown to be inhibition of MAC deposition on endothelial cells in the

Table 2. Complement-dependent disease models in which soluble CR1 reduces pathology

Complement-dependent disease models	sCR1 effects
Antibody mediated: Hyperacute rejection: Cardiac allografts in Lewis rats [88]	Allograft heart survival increased from 3.25 - / + 0.81 h to 32 - / + 4.47 h
Cardiac xenograft (guinea pigs to rats) [89, 117]	Xenograft survival increased from 17 - 23 min to 64 - 747 min in a dose-dependent manner
Immune complex-induced inflammation: Reversed passive Arthus reaction [119]	Reduces vasculitis
Immune complex-induced alveolitis [77]	Reduces alveolitis by 68 - 72%
Non-antibody mediated: Reperfusion injury: Reperfusion injury of ischemic myocardium in rats [107]	Reduces myocardial infarction size by 44%
Reperfusion injury of intestine in rats [45a]	Reduces intestinal and pulmonary injury
Thermal trauma: Acute skin and lung injury by thermal trauma [77]	Reduces damage in lung by 45 - 46% and in skin by 25% (1 hr), 44% (4 h)
sCR1, soluble CR1	

ischemic zones, and suppression of neutrophil accumulation at these sites [107]. This study demonstrated for the first time the potential utility of sCR1 as a therapeutic inhibitor of complement, and has been extended by another study showing reduction of local and remote injury after ischemia-reperfusion of the intestine [45a].

Thermal trauma. In a rat model of thermal injury to 25 - 30% of body surface area, sCR1 reduced dermal vascular permeability by 44%, and pulmonary vascular permeability and hemorrhage by 45% [77]. Thus, sCR1 is protective both locally at the primary site of non-immune injury, and distally in the lung in this model of ARDS.

Antibody-dependent complement activation

Hyperacute rejection. The current shortage of human organs available for transplantation is an incentive to develop systems in which xenogeneic organs could be used [7, 9]. However, the occurrence of natural antibodies directed to endothelial antigens in discordant xenografts [87], or of an alternative pathway activating capability of the xenograft [34, 75, 104] causes rapid rejection by a thrombotic process induced by MAC assembly on endothelial cells [13, 75, 85]. A similar process may account for hyperacute graft rejection that occurs in recipients having alloantibodies specific for donor antigens. The role of complement in this process was confirmed and a potential inhibitor described when human sCR1 was

tion of cardiac allografts in sensitized Lewis rats was delayed from 3.25 ± 0.81 h in untreated rats to 32.00 ± 4.47 h in animals that received 3 mg/kg sCR1 [88]. When administered to rats immediately prior to reperfusion of guinea pig cardiac xenografts, sCR1 extended graft survival from 17 min in untreated rats to 64 to 747 min, with the time of survival correlating with the dose of CR1 administered, ranging from 3 to 60 mg/kg in this trial [89]. Both treated and untreated animals showed significant IgM deposition in the xenografts, indicating that sCR1 inhibited damage at the level of complement activation, rather than affecting the levels of natural antibody [89, 90]. The use of recombinant RCA proteins expressed in endothelial cells of xenografts is discussed below.

Immune complex-induced inflammation. In a rat model of immune complex-induced alveolitis, sCR1 treatment reduced vascular permeability, hemorrhage and neutrophil accumulation by 68–72% [77]. sCR1 also inhibited the reversed passive Arthus reaction in rats, in which intravenous injection of ovalbumin (OVA) is followed by intradermal injection of anti-OVA antibodies [119].

Dimer forms of complement inhibitors

Systemic complement inhibition may be undesirable because of suppressed host defense; the targeting of an inhibitor to a particular tissue may overcome this drawback. In the mapping of the CR1-binding site [55], it was shown that the C3b-binding SCRs could be transferred to the amino termini of antibody heavy chains and retain function. This expression of SCRs near the antigen-binding site had no effect on the ability of the antibody to bind its hapten, 4-hydroxy-3-nitrophenacetyl, and allowed purification of the chimera on hapten-sepharose. Thus, if a monoclonal antibody specific for a particular tissue antigen unique to the site of complement activation were identified, a chimeric CR1/antibody inhibitor could be created that might yield effective local, rather than systemic complement inhibition.

Long-lasting suppression of complement activation by particular cells may be achieved through introduction into such cells of genes encoding complement regulatory proteins. This strategy may be applicable to the creation of endothelial cells having low complement-activating potential, the best example of potential utility being in xenografts because of the incompatibility between DAF, MCP, and CD59 expressed by xenografts and the heterologous source of complement [118].

To provide an experimental rationale for this approach, DAF was incorporated into porcine endothelial cells in a dose-dependent manner, and was shown to confer protection from lysis by 10% human serum [25], a result anticipated by earlier findings on the effects of DAF incorporated into PNH erythrocytes [71, 78], or expressed in murine/human cell hybrids [103]. A more prolonged effect of DAF was achieved *in vitro* by transfection of murine NIH-3T3 cells with human DAF cDNA, which suppressed their lysis by antibody and human complement [81, 110]. Similarly, high levels of expression of MCP cDNA in NIH-3T3 cells also diminished their sensitivity to classical pathway-mediated lysis [81, 110]. It is hoped that the protection *in vitro* of cells from lysis by heterologous complement may predict the successful creation of a transgenic donor animal for xenogenic transplantation [8, 25, 82, 85, 103]. However, the occurrence of humoral allo-

graft rejection, in which species-specific DAF, MCP, and CD59 [79] are incapable of suppressing complement activation induced by antibodies directed to alloantigens on graft endothelial cells suggests that the expression of these regulatory proteins in xenografts derived from transgenic animals will not avoid hyperacute rejection.

The capacity of sCR1 to suppress hyperacute rejection in a dose-dependent manner for several hours may indicate that this RCA protein should be evaluated for its potential role as a membrane inhibitor of complement activation. Accordingly, preliminary studies have demonstrated that expression of wild-type CR1 in transformed human endothelial cells confers relative resistance to lysis mediated by human complement and rabbit antibody (Braverman, Seok-Yong Kim, and Fearon, unpublished).

Conclusion

In conclusion, it is apparent that researchers are poised at the threshold of developing inhibitors of complement activation from the molecules in the RCA family. By creating soluble forms of these protective proteins for *in vivo* administration, or by making transgenic animals expressing these proteins or their derivatives, it may be possible to inhibit complement-mediated pathology stemming from autoimmune disease, reperfusion injuries, and physical trauma. This technology combined with current attempts to protect allografts from cellular rejection with monoclonal antibodies against members of the integrin family of adhesion molecules [52] makes it possible that the excessive mortality due to the severe shortage of human donor organs could be overcome by the use of xenografts.

Acknowledgement. We would like to thank Drs. Coyne and Lublin for sharing their data prior to publication.

References

1. Abrahamson DR, Fearon DT (1983) Endocytosis of the C3b receptor of complement within coated pits in human polymorphonuclear leukocytes and monocytes. *Lab Invest* 48: 162
2. Adams EM, Brown MC, Nunge M, Krych M, Atkinson JP (1991) Contribution of the repeating domains of membrane cofactor protein (CD46) of the complement system to ligand binding and cofactor activity. *J Immunol* 147: 3005
3. Ahearn JM, Fearon DT (1989) Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). *Adv Immunol* 46: 183
4. Alsenz J, Lambris JD, Schulz TF, Dierich MP (1984) Localization of the complement-component-C3b-binding site and the cofactor activity for factor I in the 38-kDa tryptic fragment of factor H. *Biochem J* 224: 389
5. Alsenz J, Schulz TF, Lambris JD, Sim RB, Dierich MP (1985) Structural and functional analysis of the complement component factor H with the use of different enzymes and monoclonal antibodies to factor H. *Biochem J* 232: 841
6. Atkinson JP, Farries T (1987) Separation of self from non-self in the complement system. *Immunol Today* 8: 212
7. Auchincloss II Jr (1988) Xenogenic transplantation. A review. *Transplantation* 46: 1
8. Bach FII (1992) Discordant xenografting: a summary and look to the future. *Trans Proc*

9. Bach FII, Turman MA, Vercellotti GM, Platt JL, Dalmaso AP (1991) Accommodation: a working paradigm for progressing toward clinical discordant xenografting. *Trans Proc* 23:205
10. Baker PJ, Osofsky SG (1980) Activation of human complement by heat-killed, human kidney cells grown in cell culture. *J Immunol* 124:81
11. Bischoff SC, De Weck AL, Dahinden CA (1990) Interleukin 3 and granulocyte/macrophage-colony-stimulating factor render human basophils responsive to low concentrations of complement component C3a. *Proc Natl Acad Sci USA* 87:6813
12. Bora NS, Lublin DM, Kumar BV, Hockett RD, Holers VM, Atkinson JP (1989) Structural gene for human membrane cofactor protein (MCP) of complement maps to within 100 kb of the 3' end of the C3b/C4b receptor gene. *J Exp Med* 169:597
13. Calne RY (1970) Organ transplantation between widely disparate species. *Trans Proc* 2:550
14. Campbell RD, Law SKA, Reid KBM, Sim RB (1988) Structure, organization and regulation of the complement genes. *Annu Rev Immunol* 6:161
15. Carel J-C, Myones BL, Frazier B, Holers VM (1990) Structural requirements for C3d/g/Epstein-Barr virus receptor (CR2/CD21) ligand binding, internalization, and viral infection. *J Biol Chem* 265:12293
16. Carroll MC, Aliquot EM, Kitzman PJ, Klickstein LB, Smith JA, Fearon DT (1988) Organization of the genes encoding complement receptors type 1 and 2, decay-accelerating factor, and C4-binding protein in the RCA locus on human chromosome 1. *J Exp Med* 167:1271
17. Carter RH, Spycher MO, Ng YC, Hoffman R, Fearon DT (1988) Synergistic interaction between complement receptor type 2 and membrane IgM on B lymphocytes. *J Immunol* 141:457
18. Cochrane CG (1984) The role of complement in experimental disease models. Springer. *Semin Immunopathol* 7:263
19. Cochrane CG, Müller-Eberhard HJ, Aikins BS (1970) Depletion of plasma complement in vivo by a protein of cobra venom: its effect on various immunologic reactions. *J Immunol* 105:55
20. Couser WG, Baker PJ, Adlers S (1985) Complement and the direct mediation of immune glomerular injury: a new perspective. *Kidney Int* 28:879
21. Cornacoff JB, Hebert LA, Smead WL, Van Aman ME, Birmingham DJ, Waxman FJ (1983) Primate-erythrocyte immune complex-clearing mechanism. *J Clin Invest* 71:236
22. Coyne KE, Hall SE, Thompson ES, Arce MA, Kinoshita T, Fujita T, Anstee DJ, Rosse W, Lublin DM (1993) Mapping of epitopes, glycosylation sites, and complement regulatory domains in human decay acceleration factor. *J Immunol* 149:2906
23. Crawford MH, Grover FL, Kolb WP, McMahon CA, O'Rourke RA, McManus LM, Pinckard RN (1988) Complement and neutrophil activation in the pathogenesis of ischemic myocardial injury. *Circulation* 78:1449
24. Daba MR, Bloem AC, Ballicux RE (1984) Immunoglobulin production by human peripheral lymphocytes induced by anti-C3 receptor antibodies. *J Immunol* 132:1197
25. Dalmaso AP, Vercellotti GM, Platt JL, Bach FH (1991) Inhibition of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor. Potential for prevention of xenograft hyperacute rejection. *Transplantation* 52:530
26. Dykman TR, Cole JL, Iida K, Atkinson JP (1983) Polymorphism of human erythrocyte C3b/C4b receptor. *Proc Natl Acad Sci USA* 80:1698
27. Dykman TR, Cole JL, Iida K, Atkinson JP (1983) Structural heterogeneity of the C3b/C4b receptor (CR1) on human peripheral blood cells. *J Exp Med* 157:2160
28. Dykman TR, Hatch JA, Atkinson JP (1984) Polymorphism of the human C3b/C4b receptor. Identification of a third allele and analysis of receptor phenotypes in families and patients with systemic lupus erythematosus. *J Exp Med* 159:691
29. Dykman TR, Hatch JA, Aqua MS, Atkinson JP (1985) Polymorphism of the C3b/C4b receptor (CR1): characterization of a fourth allele. *J Immunol* 134:1787
30. Fearon DT (1979) Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc Natl Acad Sci USA* 76:5867
31. Fearon DT (1980) Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. *J Exp Med* 152:20
32. Feasby TE, Gilbert JJ, Hahn AF, Neilson M (1987) Complement depletion suppresses Lewis rat experimental allergic neuritis. *Brain Res* 419:97
33. Fischel RJ, Bolman RM III, Platt JL, Naharian KS, Bach FH, Matas AJ (1990) Removal of IgM anti-endothelial antibodies results in prolonged cardiac xenograft survival. *Trans Proc* 22:1077
34. Forty J, Hasan R, Cary N, White DJG, Wallwork J (1992) Hyperacute rejection of rabbit hearts by human blood is mediated by the alternative pathway of complement. *Trans Proc* 24:488
35. Fujita T, Gigli I, Nussenzweig V (1978) Human C4-binding protein. II. Role in proteolysis of C4b by C3b-inactivator. *J Exp Med* 148:1044
36. Fujita T, Inoue T, Ogawa K, Iida K, Tamura N (1987) The mechanism of action of decay-accelerating factor (DAF). *J Exp Med* 166:1221
37. Garcia-Estun J, Roman RJ, Llanos EA, Garancis J (1989) Effects of complement depletion on glomerular eicosanoid production and renal hemodynamics in rat nephrotic serum nephritis. *J Lab Clin Med* 114:389
38. Gelfand MC, Frank MM, Green I (1975) A receptor for the third component of complement in the human renal glomerulus. *J Exp Med* 142:1029
39. Gelfand JA, Donelan M, Hawiger A, Burke JF (1982) Alternative complement pathway activation increases mortality in a model of burn injury in mice. *J Clin Invest* 70:1170
40. Gigli I, Fearon DT (1981) Regulation of the classical pathway convertase of human complement by the C3b receptor from human erythrocytes (abstract). *Fed Proc* 40:1172
41. Gigli I, Fujita T, Nussenzweig V (1979) Modulation of the classical pathway C3 convertase by plasma proteins C4-binding protein and C3b inactivator. *Proc Natl Acad Sci USA* 76:6596
42. Hamilton KH, Hattori R, Esmon CT, Sims PJ (1990) Complement proteins C5b-9 induce vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex. *J Biol Chem* 265:3809
43. Hebel T, Ahearn JM, Fearon DT (1991) Suppression of the immune response by a soluble complement receptor of B lymphocytes. *Science* 254:102
44. Heyman B, Wiersma EJ, Kinoshita T (1990) In vivo inhibition of the antibody response by a complement receptor-specific monoclonal antibody. *J Exp Med* 172:665
45. Heideman M (1979) Complement activation in vitro induced by endotoxin and injured tissue. *J Surg Res* 26:670
- 45a. Hill J, Lindsay TF, Ortiz F, Yeh CG, Hechtman HB, Moore FD Jr (1992) Soluble complement receptor type 1 ameliorates the local and remote organ injury after intestinal ischemia-reperfusion in the rat. *J Immunol* 149:1723
46. Hinglais N, Karatchkine MD, Mandet C, Appay MD, Bariety J (1989) Human liver Kupffer cells express CR1, CR3, and CR4 complement receptor antigens. An immunohistochemical study. *Lab Invest* 61:509
47. Holguin MI, Martin CB, Bernshaw NJ, Parker CJ (1992) Analysis of the effects of activation of the alternative pathway of complement on erythrocytes with a isolated deficiency of decay accelerating factor. *J Immunol* 148:498
48. Hourcade D, Miesner DR, Atkinson JP, Holers VM (1988) Identification of an alternative polyanion site in the human C3b/C4b receptor (complement receptor type 1) transmembrane unit and prediction of a secreted form of complement receptor type 1. *J Exp Med* 168:1255
49. Hourcade D, Holers VM, Atkinson JP (1989) The regulators of complement activation (RCA) gene cluster. *Adv Immunol* 45:381
50. Hourcade D, Miesner DR, Bee C, Zeldes W, Atkinson JP (1990) Duplication and divergence of the amino-terminal coding region of the complement receptor 1 (CR1) gene. An example of concerted (horizontal) evolution within a gene. *J Biol Chem* 265:974
51. Iida K, Nussenzweig V (1981) Complement receptor is an inhibitor of the complement

52. Isobe M, Yagita H, Okumura K, Ihara A (1992) Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* 255:1125
53. Janatova J, Reid KBM, Willis AC (1986) Involvement of disulfide bonds in the structure of complement regulatory protein C4bp. *FASEB J* 2: 8922A
54. Kalli KR, Ahearn JM, Fearon DT (1991) Interaction of iC3b with recombinant isotypic and chimeric forms of CR2. *J Immunol* 147:590
55. Kalli KR, Hsu P, Bartow TJ, Ahearn JA, Matsumoto AK, Klickstein LB, Fearon DT (1991) Mapping of the C3b-binding site in CR1 and construction of a chimeric (CR1)₂-(Fab)₂ complement inhibitor. *J Exp Med* 174:1451
56. Kazatchkine MD, Fearon DT, Appay MD, Mandel C, Barlet J (1982) Immunohistochemical study of the human glomerular C3b receptor in normal kidney and in seventy-five cases of renal diseases. *J Clin Invest* 69:900
57. Klickstein LB, Wong WW, Smith JA, Weis JH, Wilson JG, Fearon DT (1987) Human C3b/C4b receptor (CR1). Demonstration of long homologous repeating domains that are composed of the short consensus repeats characteristic of C3/C4 binding proteins. *J Exp Med* 165:1095
58. Klickstein LB, Bartow TJ, Miletic V, Rabson LD, Smith JA, Fearon DT (1988) Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1), (35) by deletion mutagenesis. *J Exp Med* 168:1699
59. Yech M, Hourcade D, Atkinson JP (1991) Sites within the complement C3b/C4b receptor important for the specificity of ligand binding. *Proc Natl Acad Sci USA* 88:4353
60. Kurimoto Y, De Weck AL, Dahinden CA (1989) Interleukin 3-dependent mediator release in basophils triggered by C5a. *J Exp Med* 170:467
61. Lennon VA, Seybold ME, Lindstrom JM, Cochrane C, Ulevitch R (1978) Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. *J Exp Med* 147:973
62. Liszewski MK, Post TW, Atkinson JP (1991) Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu Rev Immunol* 9:431
63. Lowell CA, Klickstein LB, Carter RH, Mitchell JA, Fearon DT, Ahearn JA (1989) Mapping of the Epstein-Barr virus and C3dg binding sites to a common domain on complement receptor type 2. *J Exp Med* 170:1931
64. Lozier J, Takahashi N, Putnam FW (1984) Complete amino acid sequence of human plasma β_2 -glycoprotein I. *Proc Natl Acad Sci USA* 81:3640
65. Lublin DM, Atkinson JP (1989) Decay-accelerating factor and membrane cofactor protein. *Curr Top Microbiol Immunol* 153:124
66. Lublin DM, Lemons RS, Lechman MM, Holers VM, Tykocinski ML, Medof ME, Atkinson JP (1987) The gene encoding decay-accelerating factor (DAF) is located in the complement locus on the long arm of chromosome 1. *J Exp Med* 165:1731
67. Oloko PR, Carpenter CB, Chiariello M, Fishbein MC, Radvany P, Knostman JD, Hale SL (1978) Reduction by cobra venom factor of myocardial necrosis after coronary artery occlusion. *J Clin Invest* 61:661
68. Matsumoto AK, Kopicky-Burd J, Carter RH, Tuveson DA, Tedder TF, Fearon DT (1991) Intersection of the complement and immune systems: a signal transduction complex of the B lymphocyte-containing complement receptor type 2 and CD19. *J Exp Med* 173:55
69. Medof ME, Nussenzweig V (1984) Control of the function of substrate-bound C4b-C3b by the complement receptor CR1. *J Exp Med* 159:1669
70. Medof ME, Iida K, Mold C, Nussenzweig V (1982) Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. *J Exp Med* 156:1739
71. Medof ME, Kinoshita T, Silber R, Nussenzweig V (1985) Amelioration of lytic abnormalities of paroxysmal nocturnal hemoglobinuria with decay-accelerating factor. *Proc Natl Acad Sci USA* 82:2980
72. Medof ME, Waller JL, Rutgers JL, Knowles DM, Nussenzweig V (1987) Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. *J Exp Med* 165:848
73. Merry AH, Rawlinson VI, Uchikawa M, Datta MR, Sim RB (1989) Studies on the sensitivity to complement-mediated lysis of erythrocytes (Inab phenotype) with a deficiency of DAF (decay-accelerating factor). *Br J Haematol* 73:248
74. Mitomo K, Fujita T, Iida K (1987) Functional and antigenic properties of complement receptor type 2. *CR2*. *J Exp Med* 165:1424
75. Miyagawa S, Hirose H, Shirakura R, Naka Y, Nakata S, Kawashima Y, Seta T, Matsumoto M, Uenaka A, Kitamura H (1988) The mechanism of discordant xenograft rejection. *Transplantation* 46:825
76. Müller-Eberhard HJ (1988) Molecular organization and function of the complement system. *Annu Rev Biochem* 57:321
77. Mulligan MS, Yeh CG, Rudolph AR, Ward PA (1992) Protective effects of soluble CR1 in complement- and neutrophil-mediated tissue injury. *J Immunol* 148:1479
78. Ninomiya H, Abe T, Fujita T (1989) Prolongation of red-cell life-span by incorporation of decay-accelerating factor (DAF) into red cells of paroxysmal nocturnal hemoglobinuria (PNH) (letter). *Br J Haematol* 73:132
79. Nose M, Katoh M, Okata N, Kyogoku M, Okada H (1990) Tissue distribution of HRF20, a novel factor preventing the membrane attack of homologous complement, and its predominant expression on endothelial cells in vivo. *Immunology* 70:145
80. Ogata RT, Mathias P, Bradi B, Cooper NR (1991) SCRs 1-3 of murine C4b-binding protein (C4bp) are required for binding to C4b (abstract). *Complement Inflamm* 8:202
81. Oglesby TJ, White D, Tedja I, Liszewski MK, Wright L, van den Bogarde J, Atkinson J (1991) Protection of mammalian cells from complement-mediated lysis by transfection of human membrane cofactor protein and decay-accelerating factor. *Trans Assoc Am Phys* 104:164
82. Oglesby TJ, Allen CJ, Liszewski MK, White DJG, Atkinson JP (1992) Membrane cofactor protein (CD46) protects cells from complement-mediated attack by an intrinsic mechanism. *J Exp Med* 175:1547
83. Pangburn MK (1990) Reduced activity of DAF on complement enzymes bound to alternative pathway activators. Similarity with Factor H. *Immunology* 71:598
84. Pangburn MK, Müller-Eberhard HJ (1984) The alternative pathway of complement. *Spring Semin Immunopathol* 7:163
85. Platt JL, Vercellotti GM, Dalmasso AP, Matas AJ, Bolman RM, Najarian JS, Bach FH (1990) Transplantation of discordant xenografts: a review of progress. *Immunol Today* 11:450
86. Platt JL, Vercellotti GM, Lindman BJ, Oegema TR Jr, Bach FH, Dalmasso AP (1990) Release of heparin sulfate from endothelial cells: Implications for pathogenesis of hyperacute rejection. *J Exp Med* 171:1363
87. Platt JL, Fischel RJ, Matas AJ, Reif SA, Bolman RM, Bach FH (1991) Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. *Transplantation* 52:214
88. Pruitt SK, Bollinger RR (1991) The effect of soluble complement receptor type 1 on hyperacute allograft rejection. *J Surg Res* 50:350
89. Pruitt SK, Baldwin WM III, Marsh HC Jr, Lin SS, Yeh CG, Bollinger RR (1991) The effect of soluble complement receptor type 1 on hyperacute xenograft rejection. *Transplantation* 52:868
90. Pruitt SK, Baldwin WM III, Marsh HC Jr, Lin SS, Yeh CG, Bollinger RR (1992) Effect of soluble complement receptor type 1 on natural antibody levels during xenograft rejection. *Trans Proc* 24:477
91. Quigg RJ, Cybulsky AV, Salant DJ (1991) Effect of nephritogenic antibody on complement regulation in cultured rat glomerular epithelial cells. *J Immunol* 147:838
92. Reid KBM, Day AJ (1989) Structure-function relationships of the complement components. *Immunol Today* 10:177
93. Rey-Campos J, Rubenstein P, Rodriguez de Cordoba S (1988) A physical map of the human regulator of complement activation gene cluster linking the complement genes CR1, CR2, DAF, and C4BP. *J Exp Med* 167:664
94. Reynolds M, Aubert JP, Cohen JIM, Audouin J, Tricottet V, Diebold J, Kazatchkine MD (1985) Human follicular dendritic cells express CR1, CR2 and CR3 complement receptors

95. Rodriguez de Cordoba S, Dykman TR, Ginsberg-Fellner F, Ericilla G, Aqua M, Atkinson JP, Rubinstein P (1984) Evidence for linkage between the loci encoding for the binding protein for the fourth component of human complement (C4BP) and for the C3b/C4b receptor. *Proc Natl Acad Sci USA* 81:789
96. Rodriguez de Cordoba S, Lublin DM, Rubenstein P, Atkinson JP (1985) Human genes for three complement components that regulate the activation of C3 are tightly linked. *J Exp Med* 161:1189
97. Ross GD, Lambris JD, Cain JA, Newman SL (1982) Generation of three different fragments of bound C3 with purified factor I or serum. I. Requirements for factor H vs CRI activity. *J Immunol* 129:2051
98. Scharfstein J, Ferreira A, Gigli I, Nussenzweig V (1978) Human C4-binding protein. I. Isolation and characterization. *J Exp Med* 148:207
99. Seya T, Atkinson JP (1989) Functional properties of membrane cofactor protein of complement. *Biochem J* 264:581
100. Solomkin JS, Cotta LA, Satoh PS, Hurst JM, Nelson RD (1985) Complement activation and clearance in acute illness and injury: evidence for C5a as a cell-directed mediator of the adult respiratory distress syndrome in man. *Surgery* 97:668
101. Tate CG, Uchikawa M, Tanner MJ, Judson PA, Parsons SF, Mallinson G, Anstee DJ (1989) Studies on the defect which causes absence of decay-accelerating factor (DAF) from the peripheral blood cells of an individual with the Inab phenotype. *Biochem J* 126:1489
102. Tuveson DA, Ahearn JM, Matsumoto AK, Fearon DT (1991) Molecular interactions of complement receptors on B lymphocytes: a CR1/CR2 complex distinct from the CR2/CD19 complex. *J Exp Med* 173:1083
103. Wang M-W, Wright LJ, Sims MJ, White DJG (1991) Presence of human chromosome 1 with expression of human decay-accelerating factor (DAF) prevents lysis of mouse/human hybrid cells by human complement. *Scand J Immunol* 34:771
104. Wang M-W, Johnston PS, Wright LJ, Lim SML, White DJG (1992) Immunofluorescent localization of pig complement component 3, regardless of the presence or absence of detectable immunoglobulins, in hyperacutely rejected heart xenografts. *Histochem J* 24:102
105. Watson WC, Townes AS (1985) Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-HMC loci. *J Exp Med* 162:1879
106. Weis JH, Morton CC, Bruns GAP, Weis JJ, Klickstein LB, Wong WW, Fearon DT (1987) A complement receptor locus: genes encoding C3b/C4b receptor and C3d/Epstein-Barr virus receptor map to 1q32. *J Immunol* 138:312
107. Weisman HF, Bartow T, Leppo MK, Marsh HC Jr, Carson GR, Concino MF, Boyle MP, Roux KH, Weisfeldt ML, Fearon DT (1990) Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* 249:146
108. Weiss L, Delfraissy J-F, Vazquez A, Wallon C, Galanaud P, Kazatchkine MD (1987) Monoclonal antibodies to the human C3b/C4b receptor (CR1) enhance specific B cell differentiation. *J Immunol* 138:2988
109. Whaley K, Ruddy S (1976) Modulation of the alternative complement pathway by β II globulin. *J Exp Med* 144:1147
110. White DJG, Oglesby T, Liszewski MK, Tedja I, Hourcade D, Wang M-W, Wright LJ, Wallwork J, Atkinson JP (1992) Expression of human decay-accelerating factor or membrane cofactor protein genes on mouse cells inhibits lysis by human complement. *Trans Proc* 24:474
111. Wilson JG, Tedder TF, Fearon DT (1983) Characterization of human T lymphocytes that express the C3b receptor. *J Immunol* 131:684
112. Wong WW, Farrell SA (1991) Proposed structure of the F' allotype of human CR1. Loss of a C3b binding site may be associated with altered function. *J Immunol* 146:656
113. Wong WW, Wilson JG, Fearon DT (1983) Genetic regulation of a structural polymorphism of human C3b receptor. *J Clin Invest* 72:685
114. Wong WW, Kennedy CA, Bonaccio ET, Wilson JG, Klickstein LB, Weis JH, Fearon DT (1986) Analysis of multiple restriction fragment length polymorphisms of the gene for

- human complement receptor type 1. Duplication of genomic sequences occurs in association with a high molecular mass receptor allotype. *J Exp Med* 164:1531
115. Wong WW, Cahill JM, Rosen MD, Kennedy CA, Bonaccio ET, Morris MJ, Wilson JG, Klickstein LB, Fearon DT (1989) Structure of the human CR1 gene. Molecular basis of the structural and quantitative polymorphisms and identification of a new CR1-like allele. *J Exp Med* 169:847
116. Vedeler CA, Nilsen R, Maitre R (1989) Localization of Fc receptors and complement receptors CR1 on human peripheral nerve fibers by immunoelectron microscopy. *J Neuroimmunol* 23:29
117. Xia W, Fearon DT, Moore FD Jr, Schoen FJ, Ortiz F, Kirkman RL (1992) Prolongation of guinea pig cardiac xenograft survival in rats by soluble human complement receptor type 1. *Trans Proc* 24:479
118. Yang Q-E, Want Y-Y, Bollinger RR, De Buysscher EV (1992) Discordant complement systems as a factor in hyperacute xenograft rejection. *Trans Proc* 24:481
119. Yeh GC, Marsh HC Jr, Carson GR, Berman L, Concino MF, Scesney SM, Kuestner RE, Skibbens R, Donahue KA, Ip SH (1991) Recombinant soluble human complement receptor type 1 inhibits inflammation in the reversed passive arthus reaction in rats. *J Immunol* 146:250

SYNTHETIC PEPTIDE INHIBITORS OF COMPLEMENT SERINE PROTEASES—III. SIGNIFICANT INCREASE IN INHIBITOR POTENCY PROVIDES FURTHER SUPPORT FOR THE FUNCTIONAL EQUIVALENCE HYPOTHESIS

CHARLES S. SCHASTEEN,*† R. PAUL LEVINE,‡ SHARON A. MCLAFFERTY,† RORY F. FINN,†
LEA D. BULLOCK,† JANET C. MAYDEN† and GEORGE I. GLOVER†

†Biological Sciences Department, Monsanto Company, St Louis, MO 63198, U.S.A. and

‡James S. McDonnell Department of Genetics, Washington University School of Medicine,
St Louis, MO 63110, U.S.A.

(First received 26 December 1989; accepted in revised form 6 April 1990)

Abstract—Synthetic peptides based on functionally equivalent (as defined by similar patterns of chemically equivalent amino acids) serine protease inhibitor (serpin) C-terminal sequences inhibit both classical and alternative pathways of complement activation. Inhibition was also found with hybrid peptides consisting of the cleavage site of one serpin (antithrombin III, α -1-antitrypsin, or antichymotrypsin) attached to the short and long functionally equivalent protease binding cores of the other two serpins. A hybrid peptide composed of the sequence at the site of cleavage of C4 by C1s attached to the long binding core of antithrombin III was selective in inhibiting the classical pathway with no effect on the alternative pathway at a concn of 10 μ M. Extension of the functional equivalence hypothesis has produced inhibitors of complement activation named generic + and generic -, whose sequences differ by 77% or 87%, respectively, from those of all three serpin sequences. A hybrid peptide composed of the antithrombin III cleavage site attached to the generic peptide is an inhibitor of complement activation at 500 nM, the most potent inhibitor found in this study.

INTRODUCTION

The complement system is a major line of defense against systemic bacterial infections. It is also a mediator of inflammatory responses and is responsible for the processing of immune complexes for their clearance and elimination (Porter, 1981; Porter and Reid, 1978). The activation of the complement system is largely a function of a group of serine proteases whose natural substrates are limited to specific complement proteins. Complement activation enables the initiation of events which are normally short-lived and positive in outcome; however, some disease states lead to detrimental inflammatory responses (Vogt, 1985).

The development of research tools for study of the complement serine proteases and the potential of a new therapeutic concept in the ability to regulate the activation of the classical and/or alternative complement pathways has led us to synthesize a novel series of peptide inhibitors of the complement system. Our approach is based on the homology of sequences in the C-terminal regions of the endogenous serum serine protease inhibitors (serpins) antithrombin III

(ATIII), α -1-proteinase inhibitor (antitrypsin, AT), and antichymotrypsin (ACT) (Glover and Schasteen, 1985). The homology lies both in the primary structure and in the functional equivalence of amino acid residues in their C-terminal regions. We have previously synthesized inhibitors of purified C1s of the classical pathway and purified D of the alternative pathway (Glover *et al.*, 1988) as well as inhibitors of hemolytic activity and C3a and C4a production (Schasteen *et al.*, 1988).

As described previously (Glover and Schasteen, 1985; Glover *et al.*, 1986), the functional equivalence hypothesis predicts that chemically similar amino acids are interchangeable or functionally equivalent for peptide inhibitors of complement serine proteases. Functionally equivalent amino acids are defined as hydrophobic (Trp, Phe, Tyr, Leu, Met Val, Ile), acidic (Glu, Asp), basic (Arg, Lys, His), and hydrophilic, uncharged (Gln, Asn, Ser, Thr). Amino acids considered to be distinct are Ala because of its small, hydrophobic side chain; Gly because it does not impose conformational restraints; Pro because it imposes conformational restraints; and Cys because of structural constraints that arise from its ability to form disulfide bonds. The amino acid residues contained in the C-termini of ATIII, AT, and ACT are designated by the symbols shown in the lower portion of Table 1. The patterns formed by these sequences represent both primary sequence homology and

*Author to whom correspondence should be addressed.
Nomenclature for the complement proteins is that recommended, by the World Health Organization (1970, 1981).

Table 1

A. Antithrombin III amino acid partial sequence (381-420)
 I A G R S L N P N R V T F K A N R P F L V F I R E V P L N T I I P M G R V A N P
 -ATIII

Antitrypsin amino acid partial sequence (352-394)
 P L E A I P M S I P P E V K F N K P F V F L M I E Q N T K S P L F M G K V V N P
 -AT

Antichymotrypsin amino acid partial sequence (356-399)
 T L L S A L V E T R T I V K F N R P F L M E V P T D T Q N I F P M S K V T N P
 -ACT

B.		388 ^a 403 420		LONG		Complement pathway	
VARIABLE REGION		SHORT CORE		CORE		% inhibition	
Peptide #						Peptide #	[μ M]
1						1	200
2						2	200 100 50
3						3	200
4						4	200 100
5						5	50 25
6						6	200
7						7	200 100 50
21						21	200
22						22	200 100 50
23						23	200
24						24	200
25						25	100 50
26						26	200 100
							Classical
							Alternative

amino acid functional equivalence within this region of the three serpins.

The C-terminal regions of the three serpins, ATIII, AT, and ACT each possess a core binding region within which a short core (identified as residues 388-403 of ATIII) and a long core (identified as residues 388-420 of ATIII) can be distinguished. It was assumed that these segments of homology are involved in serpin/serine protease binding. Each naturally occurring serpin also has a variable region within which cleavage by its target serine protease occurs. Localization of the cleavage site (indicated by

the arrows below the sequences given in Table 1) within the variable region rather than in the functionally homologous core regions is consistent with the importance of the cleavage site in determining the specificity of each serpin for its target enzyme.

Further evidence for the functional equivalence hypothesis is presented here with inhibitors of the complement system which are hybrid peptides of various cleavage sites used by proteases attached to the short and long cover peptides of ATIII, AT and ACT. Included is a hybrid peptide of the fourth complement protein (C4) cleavage site attached to

the long inhibitor functional validated sequence the ability among the comple

Synthetic

Peptide

(Stewart

reverse-p

or C18 2

sequences

and sequ

was con

spectrom

short cor

short an

Cys418 c

oxidation

deblocki

addition

length of

the long

activities

For ease

this pap

in other

tables is

et al., 19

Prepara

Antib

prepare

hemolys

describ

stored

pH 7.5

gelatin

(ER) w

veins of

Farm,

contain

Hemoly

Class

by incu

known

and m

(Harris

saline

(solubili

saline

synthe

compo

a total

the long core of ATIII. This peptide is a selective inhibitor of the classical complement pathway. The functional equivalence hypothesis has been further validated by synthesis of peptides that have little sequence identity with ATIII, AT, or ACT, but retain the ability to inhibit complement activation. Included among these peptides is a novel sequence that inhibits complement activation at a concn of 500 nM.

MATERIALS AND METHODS

Synthetic peptides

Peptides were prepared by the solid-phase method (Stewart and Young, 1984) and were purified by reverse-phase semi-preparative chromatography (C4 or C18 25 cm × 10 mm, Vydac, Hyperius, CA). The sequences were confirmed by amino acid composition and sequence analysis. Identity of certain peptides was confirmed by fast atom bombardment mass spectrometry. Ile was substituted for Met in the AT short cores, Leu was substituted for Met in all other short and long cores and Ala was substituted for Cys418 of ATIII to avoid difficulties resulting from oxidation of the side chains during synthesis and deblocking. The generic peptide was extended an additional three amino acids to match the actual length of AT; addition of the three amino acids to the long cores had no major effect on inhibitory activities in cases where the comparison was made. For ease of relating the activities of the peptides in this paper to other activities of the same peptides in other papers, the numbering of the peptides in the tables is according to that used previously (Glover *et al.*, 1988; Schasteen *et al.*, 1988).

Preparation of cells

Antibody sensitized sheep erythrocytes (EA) were prepared by incubating sheep erythrocytes (E) with hemolysin (PEL-Freeze Biologicals, Rogers, AR) as described (Harrison and Lachman, 1986). EA were stored at 4°C in 5.6 mM veronal buffered saline, pH 7.5, containing 2.5% w/v dextrose, 0.05% w/v gelatin, and 0.01% sodium azide. Rabbit erythrocytes (ER) were obtained from blood drawn from the ear veins of New Zealand white rabbits (Boswell's Rabbit Farm, St Louis, MO) into cold Alsevers' buffer containing 0.01% sodium azide.

Hemolytic assays

Classical pathway hemolytic activity was measured by incubating titrated normal human serum (NHS) of known hemolytic activity with EA for 1 hr at 37°C and measuring absorbance at 414 nm as described (Harrison and Lachman, 1986). Phosphate buffered saline (PBS) was used as buffer because of the lower solubility of peptide inhibitors in veronal buffered saline. A typical classical pathway experiment with synthetic peptide inhibitors contained the following components (final concn) added in sequence to give a total volume of 250 µl: 100 µl PBS⁺⁺ (PBS contain-

ing 0.15 M CaCl₂ and 0.5 M MgCl₂) with variable concns of synthetic peptide inhibitor or no peptide in the control, 100 µl NHS (final dilution was from 1:100 to 1:120), and 50 µl EA (2.5 × 10⁷ cells washed twice with PBS⁺⁺) were added last to initiate the activation. The dilution of NHS was such that control hemolysis was 75–85%. Incubations were carried out for 60 min at 37°C after which 0.75 ml of cold PBS was added to stop hemolysis and the preparations were centrifuged for 5 min at 5000 g. A sample of the supernate was removed and its absorbance at 414 nm measured in a Zeiss model PM20L spectrophotometer. Alternative pathway hemolytic assays were carried out in the same manner as for the classical pathway except that EA were replaced with ER. Dilution of NHS required for a control hemolysis of 75–85% was 1:10 in PBS⁺⁺.

RESULTS AND DISCUSSION

Numerous investigators have suggested the importance of drugs that could control complement mediated processes (Patrick and Johnson, 1980; Asghar, 1984; Bailey *et al.*, 1985; Vogt, 1985). Diseases in which complement activation is known to occur, e.g. myasthenia gravis, rheumatoid arthritis, serum sickness, hemolytic anemias, and systemic lupus erythematosus (Vogt, 1985) are possible targets for therapeutic intervention with complement inhibitors. Inhibitors for both classical (Asghar, 1984; Boackle *et al.*, 1979; Takada *et al.*, 1978; Caporale *et al.*, 1981; Andreatta *et al.*, 1980) and alternative (Kaneko *et al.*, 1980) complement pathways have been described; however, many of these require relatively high concns (10–10,000 µM) for inhibition of complement activation both *in vivo* and *in vitro* (Patrick and Johnson, 1980). Complement activation is the result of sequential proteolysis by serine proteases with trypsin-like specificity, so one approach to blocking complement activation is inhibition of one or more key complement proteases. Protease inhibitors are commonly based on the sequences of the physiological substrate site cleaved by the target protease since the natural substrates of the target proteases are expected to have high affinity for the enzymes that normally cleave them (Sturtzebecher, 1984). This has not been a productive rationale for the design of inhibitors of the complement system: a peptide encompassing the cleavage site of C3 is a poor inhibitor of the C3 convertases (Andreatta *et al.*, 1981; Schasteen *et al.*, 1988) and a peptide encompassing the site in factor B cleaved by Factor D does not inhibit this enzyme (Glover *et al.*, 1988). A second approach to inhibition of serum serine proteases is based on the functional equivalence of the C-termini of serpins and has yielded inhibitors of complement activation (Glover and Schasteen, 1985; Glover *et al.*, 1988) and of the production of C4a and C3a (Schasteen *et al.*, 1988). Functionally equivalent C-termini of ATIII, AT and ACT are evident in the

similar pattern of chemically equivalent amino acids illustrated in Table 1A. Further evidence in support of the hypothesis that these functionally equivalent regions are common protease binding domains of serpins is presented below.

Inhibition of hemolytic activity by hybrid peptides based on the cleavage sites and short cores of ATIII, AT, and ACT

Peptides from the C-termini of serpins on which the functional equivalence hypothesis is based can be described as a cleavage site (variable for different target proteases) and a short or long core region as illustrated in Table 1B for ATIII. The cleavage site is represented by the region flanking the peptide bond cleaved by the target protease, e.g. Arg384-Ser385 of ATIII, Met358-Ser359 of AT and Leu358-Ser359 of ACT (indicated by the arrows below the sequences). A peptide encompassing only the cleavage site of ATIII, 1, is a poor inhibitor of either complement pathway (Table 1B). Peptides encompassing only the short core of ATIII, 2, or ACT, 4, inhibit complement activation of both pathways. The short core of AT, 3, however, is not an inhibitor of complement activation at a concn of 200 μ M. This trend toward decreased effectiveness continues when the AT short core is extended to include the cleavage site, 6, whereas extension of either ATIII or ACT, 2 and 4, respectively, increases the inhibitory potency, 5 and 7. The hybrid peptide composed of the cleavage site of AT attached to the short core of ATIII, or AT/ATIII, 21, is not an inhibitor of complement activation whereas the hybrid ACT/ATIII short core, 22, is an inhibitor although not as effective as the natural ATIII/ATIII short core sequence, 5. The trend in which the AT sequence is a relatively poor complement inhibitor continues with hybrid peptides 23 and 24, in which the ATIII and ACT cleavage sites are attached to the AT short core. Hybrids of AT and ATIII cleavage sites attached to ACT short core, 25 and 26, respectively, provide inhibitors of both complement pathways. These results are consistent with the hypothesis that the core peptide contributes mainly to binding to the proteases. The hypothesis predicts that all three cores are functionally equivalent and should be inhibitors. The AT core, however, was not an inhibitor of complement activation at the 200 μ M concentration tested, suggesting that the functional equivalence hypothesis may be generally applicable, but that primary sequence still plays a significant role in certain cases. This is reasonable since target proteases differ significantly in actual amino acid sequence and thus it is expected that the actual sequence of the inhibitor might be important.

Inhibition of hemolytic activity by hybrid peptides based on the cleavage sites and long cores of ATIII, AT, and ACT

The inhibition of hemolytic activity by a series of hybrid peptides extended to include a second region

of functional equivalence (long core) is shown in Table 2A. Peptide 27, representing the second functionally equivalent region of ATIII alone is not a inhibitor of complement activation; however, when attached to the short core of ATIII to give the ATIII long core (illustrated for ATIII in Table 2B), 8, an inhibitor of complement activation more potent than at ATIII short core, 2, is obtained. A scrambled sequence of the ATIII long core, 46, lacks inhibitory activity and shows the importance of the pattern of functional equivalence in the primary sequence. The long cores of all three serpins are inhibitors of complement activation, ATIII, 8; AT, 9; ACT, 10. Protease inhibition activity for complement, tPA (Schasteen *et al.*, 1986), and coagulation (Schasteen *et al.*, unpublished data) seems to be inherent in the long cores of functional equivalence. These results offer strong support for the hypothesis that these are functionally equivalent protease binding regions. The general trend noted above for an increase in inhibitor potency by addition of the cleavage site is seen with ATIII, 11, and ACT, 13; however, AT, 12, resumes the trend seen with the short core AT peptides of being ineffective as complement inhibitors at the 200 μ M concn tested. Hybrid peptides of AT and ACT cleavage sites with the long ATIII core, 28 and 29, respectively, are complement inhibitors with no significant increase in potency over the ATIII long core alone, 8. The importance of the cleavage site in the potency of an inhibitor is shown by comparison of the AT sequence, 12 with the hybrid peptides of ATIII and ACT cleavage site attached to the AT long core, 30 and 31, respectively. ATIII, 30, and ACT, 31, cleavage sites, but not the AT cleavage site, 12, attached to the AT cores are complement inhibitors. The ATIII cleavage site attached to the ACT long core, 32, provides the most effective complement inhibitor of this series whereas the AT cleavage site attached to the long core of ACT, 33, is a marginally effective inhibitor of the classical pathway and ineffective as an inhibitor of the alternative pathway at the 200 μ M concn tested.

Further evidence in support of the functional equivalence hypothesis

If this hypothesis is valid, substitution of chemically equivalent amino acids in the regions of significant homology (Table 3; long or short cores) will markedly change the primary sequence, but protease inhibitory activity should be retained. Results obtained with the long core peptides, 8, 9, and 10 are included for reference in Table 3B. The generic sequence, 34, contains only seven amino acids that are identical in the primary sequences of ATIII, AT and ACT, but is 77% different from the three serpin sequences (Table 3A). The novel (not found in nature) generic peptide, 34, is indeed an inhibitor of complement activation (Table 3B). The addition of the AT cleavage site to the generic core, 36, results in a peptide with

Table 2

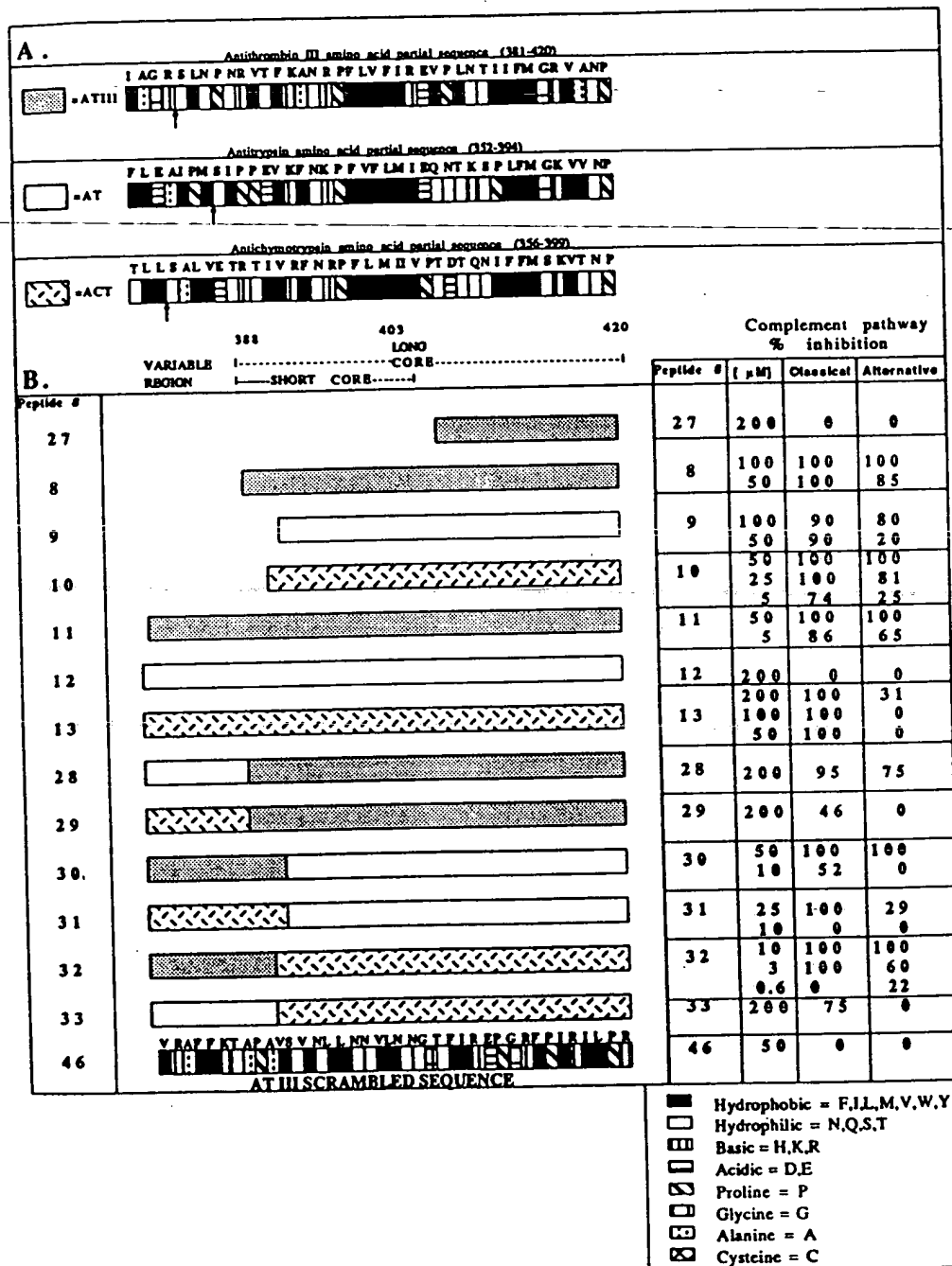


Table 3

Table 3					
Antithrombin III amino acid partial sequence (381-423)					
A.					
I A G R S L N P N R V T F K A N R P F L V F I R E V P L N T I I P M G R V A N P C V K					
-ATIII					
Antitrypsin amino acid partial sequence (352-397)					
F L E A I P M S I P P E V E F N K P P V F L M I E Q N T K S P L F M G K V V N P T Q K					
-AT					
Antichymotrypsin amino acid partial sequence (332-402)					
T L L S A L V E T R T I V R F N R P F L M H V P T D T Q N I F F M S K V T N P S K P					
-ACT					
Generic sequence (1-31)					
L R Y N K P F I L V L P R T P O N S L V F L O R I S N P A T K					
-Generic					
Generic + sequence (1-31)					
Y K L Q R G L V I L V L D S G O T Q V P I V T K L N Q O T S K					
-Generic +					
B.					
Complement pathway % inhibition					
Peptide #	Peptide	Peptide #	[μM]	Classical	Alternative
8		8	100 50	100 100	100 85
9		9	100 50	100 100	100 85
10		10	50 25 5	100 100 74	100 81 25
34		34	100 25 10	100 77 2	83 2 0
35		35	10 1 0.5	100 100 82	100 35 25
36		36	200	0	0
37		37	100	99	45
38		38	50	77	39
		<div>■ Hydrophobic = F, I, L, M, V, W, Y</div> <div>□ Hydrophilic = N, Q, S, T</div> <div>▢ Basic = H, K, R</div> <div>▣ Acidic = D, E</div> <div>▤ Proline = P</div> <div>▥ Glycine = G</div> <div>▦ Alanine = A</div> <div>▧ Cysteine = C</div>			

no inhib generic c other A' to the ge or decre system. inhibitic of the / core, 35 inhibito Comple peptide (82%) (This dr out the underst found b generic of funct cleavag underst proteas greater retains from th acids o comple

Eas

no inhibitory activity even though it retains the generic core peptide consistent with results for most other AT peptides. The ACT cleavage site attached to the generic core, 37, does not significantly increase or decrease the ability to inhibit the complement system. This contrasts with the dramatic increase in inhibition seen with the hybrid peptide comprised of the ATIII cleavage site attached to the generic core, 35, which provides the most effective peptide inhibitor of complement activation in this study. Complete inhibition of the alternative pathway by peptide 35 is seen at $10 \mu\text{M}$ and significant inhibition (82%) of the classical pathway is seen at 500 nM . This dramatic increase in inhibitory activity points out the intricacies of this system which are still not understood. The magnitude of the inhibitory range found by attachment of different cleavage sites to the generic core suggests that, although the hypothesis of functional equivalence appears generally valid, the cleavage site plays a significant, yet incompletely understood, role in the inhibition of the complement proteases. The generic + peptide, 38, represents a greater departure from serpin sequence identity, but retains functional equivalence (Table 3A). It differs from the serpin sequences at all but four basic amino acids or by 87%, yet retains the ability to inhibit complement activation.

Importance of the cleavage site to the ability to inhibit complement activation

The importance of the cleavage site in determining inhibitory potency is further demonstrated in Table 4. We have attached the thrombin cleavage site of the α -chain of fibrinogen and the C1s cleavage site of C4 to either short or long cores of ATIII. The hybrid peptide of thrombin cleavage site on α -fibrinogen coupled to the short core of ATIII, 16, was reported previously to increase the inhibitory potency over the ATIII short alone with respect to complement inhibition (Schasteen *et al.*, 1988). The thrombin cleavage site on α -fibrinogen also increases the potency of the long core of ATIII, 39 vs 8; the effect of the fibrinogen cleavage site is comparable to that of the ATIII cleavage site, 11 (Table 2B). We have also reported finding that the C4 cleavage site attached to the short core peptide of ATIII, 14, confers significant selectivity in inhibiting the classical pathway over the alternative pathway of complement activation. This is confirmed in the hybrid C4/ATIII long core peptide, 40, which shows complete inhibition of the classical pathway at $25 \mu\text{M}$ with no effect on the alternative pathway of complement activation. This is significant as use of this peptide would potentially control classical pathway activation while allowing the alternative pathway to perform unimpeded.

Table 4

Antithrombin III amino acid partial sequence (382-420)			
	382		420
	AGRELNPNRVTFKANEFFLVFIREVPLNTIIFMGKVANP		
	LONG CORE		
	SHORT CORE		
Peptide #		INHIBITION OF HEMOLYTIC ACTIVITY (%)	
		μM	
		CLASSICAL	ALTERNATIVE
2		200	59
		100	8
16		50	100
		25	61
14		25	0
8		100	100
		50	85
39		10	97
		5	45
		1	0
40		50	0
		25	0
		10	0

Methionine 414 was substituted by leucine and Cysteine 417 was substituted by alanine for ease of synthesis and purification


Numbering of peptides 1-17 are from preceding papers (Glover *et al.*, 1988; Schasteen *et al.*, 1988)

- Hydrophobic = F,I,L,M,V,W,Y
- Hydrophilic = N,Q,S,T
- Basic = H,K,R
- Acidic = D,E
- Proline = P
- Glycine = G
- Alanine = A
- Cysteine = C








Table 5









Antithrombin III amino acid partial sequence

381 403
IAGRLNPNRVTIKANRPFLVFI
CLEAVAGE SITE SHORT CORE

 = ATIII

Complement pathway
% inhibition

Peptide#		[μ M]	Classical	Alternative
2	390 403 	200 100	100 11	59 8
14	75 82 390 403 COMPLEMENT C1 LQRALEIL 	25	40	0
41	68 79 390 403 COMPLEMENT C1 RDKGQAQLRAL 	100	39	16
42	68 83 390 403 COMPLEMENT C1 RDKGQAQLQRALEILQ 	50	82	94
43	68 87 390 403 COMPLEMENT C1 RDKGQAQLQRALEILQEEDL 	50	0	0
44	68 91 390 403 COMPLEMENT C1 RDKGQAQLQRALEILQBEDLIDED 	100	0	0
45	68 95 390 403 COMPLEMENT C1 RDKGQAQLQRALEILQBEDLIDEDDIPV 	50	0	0

 Hydrophobic = F,I,L,M,V,W,Y
 Hydrophilic = N,Q,S,T
 Basic = H,K,R
 Acidic = D,E
 Proline = P
 Glycine = G
 Alanine = A
 Cysteine = C

* Numbering of peptides 1-17 are from preceding papers (Glover et al., 1988; Schaefer et al., 1988).

* Numbering of peptides 1-17 are from preceding papers (Glover *et al.*, 1988; Schastee *et al.*, 1988).

■ Hydrophobic = F,I,L,M,V,W,Y
 □ Hydrophilic = N,Q,S,T
 ▨ Basic = H,K,R
 ▩ Acidic = D,E
 ▤ Proline = P
 ▥ Glycine = G
 ▦ Alanine = A
 ▧ Cysteine = C

Effect of spacing between cleavage sites and cores on inhibition of complement activation

The importance of the spacing of a cleavage site from the core peptide is illustrated in Table 5. Peptides 41-45 represent increasing or decreasing distance between the cleavage site on C4 and the short core of ATIII. The original cleavage site attachment in 14 was an arbitrary choice based on placing the cleavage site of C4 in the same position relative to the cleavage site in ATIII. Moving the cleavage site two amino acid residues closer to, 41, or farther from, 42, the short core significantly lowers the inhibitory potency and reduces selectivity for the classical pathway. Moving the cleavage site of C4 in four amino acid blocks relative to peptide 42, 43-45, suggests that the distance between the short core and the cleavage site in 14 is essential to retain inhibitory activity.

Effect of peptides from the C-terminal portion of ATIII on complement activation

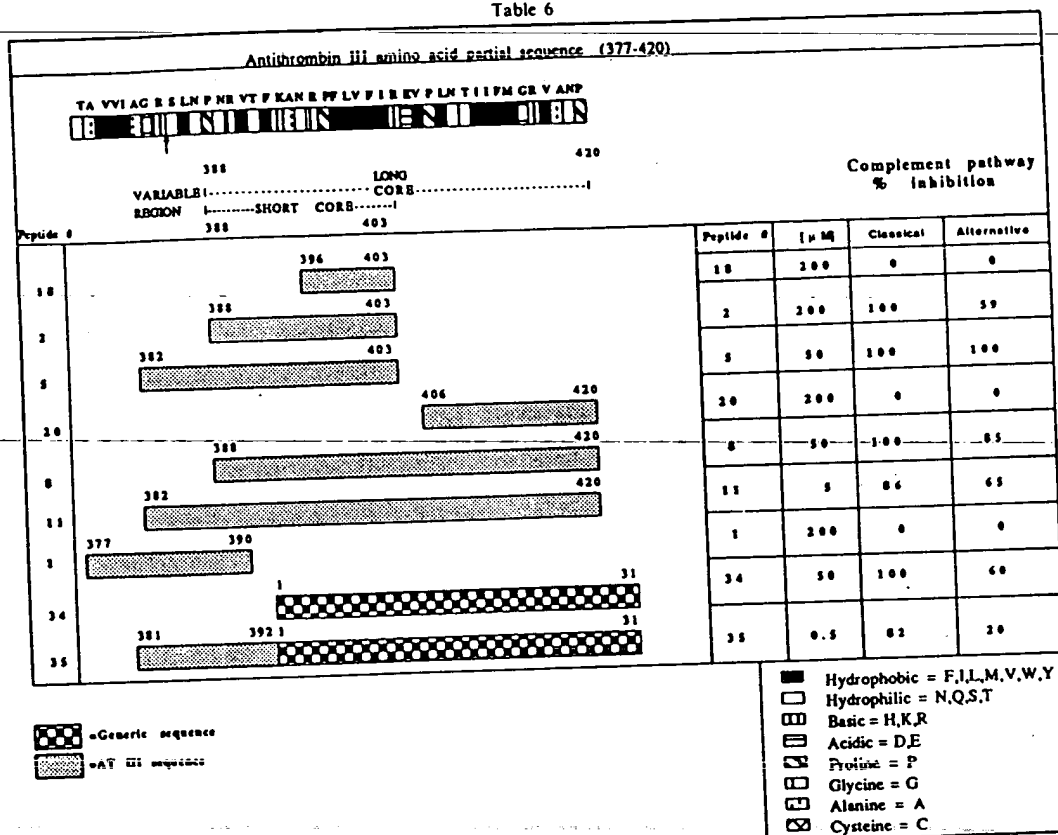
The data from the individual peptides shown in Table 6 has been presented elsewhere in this paper; however the order of presentation in this table points out special features of the structure-function relation-

ship of complement inhibition. Peptide 18 represents half the short core of ATIII and is not active as an inhibitor of complement activation at 200 μ M. The entire short core peptide of ATIII, 2, inhibits both pathways of complement activation at a concentration of 200 μ M. Extension of the short core through the cleavage site of ATIII gives peptide 5, which is an effective complement inhibitor at 50 μ M showing an apparent 4-fold increase in inhibitory activity. This result shows that addition of the cleavage site can increase the inhibitory potency of the short core of ATIII for inhibition of the complement cascade. Peptide 20 represents half of the long core of ATIII, and this peptide is not an inhibitor of complement activation at 200 μ M. Addition of the short core region to 20 results in the long core region, 8, which is an effective complement inhibitor at a 50 μ M concn. Addition of the cleavage site region of ATIII (by itself not an inhibitor of complement activation as shown by 1) to the long core of ATIII provides peptide 11, an effective inhibitor of complement activation at 5 μ M, a 10-fold increase in inhibitory potency over the long core of ATIII alone and further evidence of the importance of the cleavage site region. The generic peptide, 34, is an inhibitor of complement activation at 50 μ M

and the a
35, an in
of 500 n
inhibitory

The da
(Glover
Schastee
in suppo
sequence
constitut
class of
from the
complem
alternativ
proteases
strates. F
and gen
identity
inhibit co
35 consid
the gene
complem
ally hom
ing diffe
possible
Most A
activation
the hybr
ACT pe
core inh

Table 6



and the addition of the ATIII cleavage site provides 35, an inhibitor of complement at a concentration of 500 nM, a 100-fold apparent increase in the inhibitory potency over the generic core alone.

The data presented in this and previous reports (Glover and Schasteen, 1985; Glover *et al.*, 1988; Schasteen *et al.*, 1986 and 1988) provide evidence in support of the hypothesis that the C-terminal sequences of serpins are functionally equivalent and constitute a common protease binding domain in this class of proteins. Functionally equivalent peptides from the C-termini of ATIII, AT and ACT inhibit complement activation in both classical and alternative pathways and the action of complement proteases D and C1s on their natural protein substrates. Further, novel functionally equivalent generic and generic + peptides having minimal sequence identity to the serpins, ATIII, AT, and ACT, also inhibit complement activation and the hybrid peptide 35 consisting of the ATIII cleavage site attached to the generic peptide, is the most potent inhibitor of complement activation found in this study. Functionally homologous peptides are not equipotent reflecting differences in sequences; to date it has not been possible to establish a structure-potency relationship. Most AT peptides are not inhibitors of complement activation; the exceptions being the long core 9 and the hybrid peptides 26, 28, 30, and 31. All ATIII and ACT peptides containing a complete short or long core inhibit complement activation. There are rare

exceptions among hybrid peptides containing AT sequences such as peptides 21, 23, 24 and 36.

Although not completely understood, it is significant that selective inhibitors of the classical pathway of complement activation have been discovered. Hybrid peptides consisting of the C1s cleavage site of C4 fused to long or short cores are selective inhibitors of classical pathway activation. The selectivity of inhibitors of the classical pathway is also noted when the ACT cleavage site is attached to the ATIII or ACT long core (i.e. compare the effects of peptide 8 vs 29 and peptide 13 vs 10). This observation is important in that it supports inhibition of specific steps in these activation pathways by these peptides rather than blocking hemolysis in a nonproteolytic step common to both pathways. The results of the studies reported here on inhibition of complement proteases and complement activation lend support to the hypothesis of the functional equivalence of these serpin sequences. The inhibitors discovered provide useful biochemical tools for studying the role of complement in complex biochemical systems and may provide leads to therapeutics based on inhibition of complement activation by both pathways or by the classical pathway alone.

REFERENCES

- Andreatta R. H., Rahuel J., Wesp M. and Dukor P. (1981) Attempts at specific inhibition of C3-convertase. In *Enzyme Inhibitors* (Edited by Brodbeck U.), pp. 261-272. Verlag Chemie, Basel.

- Asghar S. S. (1984) Pharmacological manipulation of complement system. *Pharmac. Rev.* **36**, 223-244.
- Bailey D. M., DeGrazia G. D., Alexander E. J., Powles R. G., Johnson R. E., Patrick R. A., Heerdt B. G., Fairbairn M. E. and Pruden D. J. (1985) Syntheses and complement inhibitory activities of 4-(2-phenyl-1H-indol-3-yl)cyclohexane-1-carboxylic acids. *J. med. Chem.* **28**, 160-164.
- Boackle R. J., Johnson B. J. and Caughman G. B. (1979) An IgG primary sequence exposure theory for complement activation using synthetic peptides. *Nature (Lond.)* **282**, 742-743.
- Caporale L. H., Gaber S.-S., Kell W. and Gotze O. (1981) A fluorescent assay for complement activation. *J. Immun.* **126**, 1963-1965.
- Glover G. I. and Schasteen C. S. (1985) A generic approach to serine protease inhibitors. *Fedn. Proc. Fedn. Am. Soc. exp. Biol.* **45**, 1858 (Abstract 2206).
- Glover G. I., Schasteen C. S., Liu W.-S. and Levine R. P. (1988) Synthetic peptide inhibitors of complement serine proteases—I. Identification of functionally equivalent protease inhibitor sequences in serpins and inhibition of C1s and D. *Molec. Immun.* **25**, 1261-1267.
- Harrison R. A. and Lachmann P. J. (1986) Complement technology. *Handbook of Experimental Immunology*, Vol. 1, *Immunochemistry* (Edited by Weir D. M., Herzenberg L. A. and Blackwell C.), pp. 39.1-39.49. Blackwell Scientific, Oxford.
- Kaneko I., Fearon D. T. and Austen K. F. (1980) Inhibition of the alternative pathway of human complement *in vitro* by a natural microbial product, complementin. *J. Immun.* **124**, 1194-1198.
- Patrick R. A. and Johnson R. E. (1980) Complemental inhibitors. In *Annual Reports in Medicinal Chemistry*, Vol. 15 (Edited by Hess H.-J. and Bailey D. M.), pp. 192-201. Academic Press, New York.
- Porter R. R. (1981) The proteolytic enzymes of the complement system. In *Methods in Enzymology*, Vol. 80, *Proteolytic Enzymes*, Part C (Edited by Lorand L.), pp. 3-5. Academic Press, New York.
- Porter R. R. and Reid K. B. M. (1978) The biochemistry of complement. *Nature (Lond.)* **275**, 699-704.
- Schasteen C. S., McLafferty S. A., Glover G. I., Han C. Y., Mayden J. C., Liu W.-S. and Levine R. P. (1988) Synthetic peptide inhibitors of complement serine proteases—II. Effects on hemolytic activity and production of C3a and C4a. *Molec. Immun.* **25**, 1269-1275.
- Schasteen C. S., Manning P., McLafferty S., Radosevich J., Finn R., Glover G. and Tiemeier D. (1986) Inhibition of fibrinolytic activity *in vitro* with synthetic peptides. *Fed. Proc.* **45**, 966 (Abstract 4720).
- Stewart J. M. and Young J. D. (1984) *Solid Phase Peptide Synthesis*. 2nd edn. Pierce Chemical Co., Rockford, IL.
- Sturzebecher J. (1984) Inhibitors of thrombin. In *The Thrombin*, Vol. I (Edited by Machovich R.), pp. 131-160. CRC Press, Boca Raton.
- Takada Y., Arimoto Y., Minoda H. and Takada A. (1978) Inhibition of the classical and alternative pathways by amino acids and their derivatives. *Immunology* **34**, 509-515.
- Vogt W. (1985) Drugs and the complement system. *Trends Pharm. Sci.* **6**, 114-119.
- World Health Organization (1970) Nomenclature of complement. *Immunochemistry* **7**, 137-142.
- World Health Organization (1981) Nomenclature of the alternative activating pathway of complement. *Bull. W.H.O.* **59**, 489-491.

Depa

Abstr
affini
comp
posse
germ
quen
reass
chair
relate
quen
idiot
obse
in in
howe
subst
20.5°
Arg
(~10
H. cl

The comple
interactions
body (Ab) a
beginning t
various hap
primary str
binding dif
involved in
not determ
were directl
that second
Although r
various ha

*Supported
Research
U.S.A.

†To whom
Abbreviation
purified;
fluoresce
heavy cl
i.p., intr
Mab, m
saline; s
sodium c
esis; V_H
variable

DOCUMENT-IDENTIFIER: US 6232296 B1

TITLE: Inhibition of complement activation and complement modulation by use of modified oligonucleotides

BSPR:

The complement cascade consists of two major branches, the classical and alternative pathways. Though these pathways are initiated differently, they converge at the step of complement protein C3 activation (see FIG. 5). The complement cascade can mediate undesirable cellular damage in inflammatory, immune or autoimmune (auto-antibody-mediated) conditions such as; myasthenia gravis, immune complex excess syndromes such as systemic lupus erythematosus, ischemia-reperfusion states, hyper-acute rejection of transplants, organ failure conditions such as adult respiratory distress syndrome, Alzheimer's disease and related neurodegenerative disorders, among others.

BSPR:

Candinas et al., describe the activation and depletion of complement by using cobra venom factor in conjunction with a recombinant soluble complement receptor type 1 protein (sCR1), and the use of such molecules in treating hyperacute xenograft rejection. (Candinas, D. et al., Transplantation 1996 15;62(3):336-342) sCR1 is a recombinant protein that has been shown to inhibit both the classical and alternative pathways of complement and thereby limits the production of proinflammatory products such as the anaphylatoxins (complement proteins C3a, C4a and C5a). sCR1 has also been described by Moore, F D Jr., as the first protein useful to treat adverse clinical situations which are complement-dependent, and further describes potential uses for sCR1 to treat thermal injury, ARDS, septic shock, and ischaemia/reperfusion injury events such as myocardial infarction after thrombolytic therapy.

(Moore, F-D
Jr., Adv. Immunol 1994 56:267-299) U.S. Pat. No. 5,856,297,
Fearon et al.,
issued Jan. 5, 1999 claims pharmaceutical compositions
comprising a CR1
protein in various modifications and describes CR1 and the
recombinant forms of
the protein as being useful in the diagnosis and treatment of
disorders
involving complement activity and inflammation.

DEPR:

As described above, various methods have been investigated to
inhibit
complement activation. Wuillemin et al., J. Immunol
15;159(4):1953-60, (1997),
describes the use of glycosaminoglycans, such as heparin, to
inhibit the
interaction of complement component C1q with other complement
activators and
the assembly of the classical and alternative pathway C3
convertases.
Naturally occurring glycosaminoglycans such as dextran sulfates,
heparin,
N-acetylheparin, heparan sulfate, dermatan sulfate, and
chondroitin sulfates A
and C were studied to determine their effectiveness at inhibiting
the
deposition of C4 and C3 on immobilized aggregated human IgG and
to reduce fluid
phase formation of C4b/c and C3b/c. In the study it was
concluded that
glycosaminoglycans such as the low molecular weight dextran
sulfate (m.w.
5000) may serve as candidates for pharmacological manipulation of
complement
activation via potentiation of C1 inhibitor.

DEPR:

By "abnormal and/or undesirable conditions" is meant any
conditions that have
an inflammatory, immune or autoimmune component associated with
the activation
of the complement cascade. An abnormal and/or undesirable
condition can be,
but is not limited to: myasthenia gravis, immune complex excess
syndromes such
as systemic lupus, erythematosus, ischemia-reperfusion states,
angioedema,
hyper-acute rejection of transplants, organ failure conditions
such as adult

respiratory distress syndrome, Alzheimer's disease and related neurodegenerative disorders. Such conditions are generally determined by registered physicians.



US006232296B1

(12) **United States Patent**
Henry

(10) Patent No.: **US 6,232,296 B1**
(45) Date of Patent: **May 15, 2001**

(54) **INHIBITION OF COMPLEMENT
ACTIVATION AND COMPLEMENT
MODULATION BY USE OF MODIFIED
OLIGONUCLEOTIDES**

(75) Inventor: **Scott Henry, Cardiff, CA (US)**

(73) Assignee: **Isis Pharmaceuticals, Inc., Carlsbad,
CA (US)**

16(7-9), 1673-1676 (1997).*

Henry, S.P. et al., Activation of the Alternative Pathway of Complement by a Phosphorothioate Oligonucleotide: Potential Mechanism of Action, *J. Pharmacol. Exp. Ther.* 281 (2), 810-816 (1997).*

Merriam Webster's Collegiate Dictionary, Tenth Edition, at p. 541-542 (1996).*

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/409,816**

(22) Filed: **Sep. 30, 1999**

(51) Int. Cl.⁷ **A61K 48/00; C12Q 1/68;
C12P 19/34; C12N 15/85; C07H 21/04**

(52) U.S. Cl. **514/44; 435/6; 435/91.1;
435/325; 435/363; 435/375; 536/23.1**

(58) Field of Search **435/6, 91.1, 325,
435/363, 366, 375; 514/44; 536/23.1**

Henry, Scott P., et al., "Activation of the Alternative Pathway of Complement by a Phosphorothioate Oligonucleotide: Potential Mechanism of Action", *The Journal of Pharmacology and Experimental Therapeutics* 1997 vol. 281, No. 2, 810-816.

* cited by examiner

Primary Examiner—John L. LeGuyader

Assistant Examiner—Mark L. Shibuya

(74) Attorney, Agent, or Firm—Licata & Tyrrell P.C.

(56) References Cited

FOREIGN PATENT DOCUMENTS

WO 95/32719 5/1995 (WO) .

WO 95/32719

A1 * 12/1995 (WO) .

WO 97/07354 5/1997 (WO) .

OTHER PUBLICATIONS

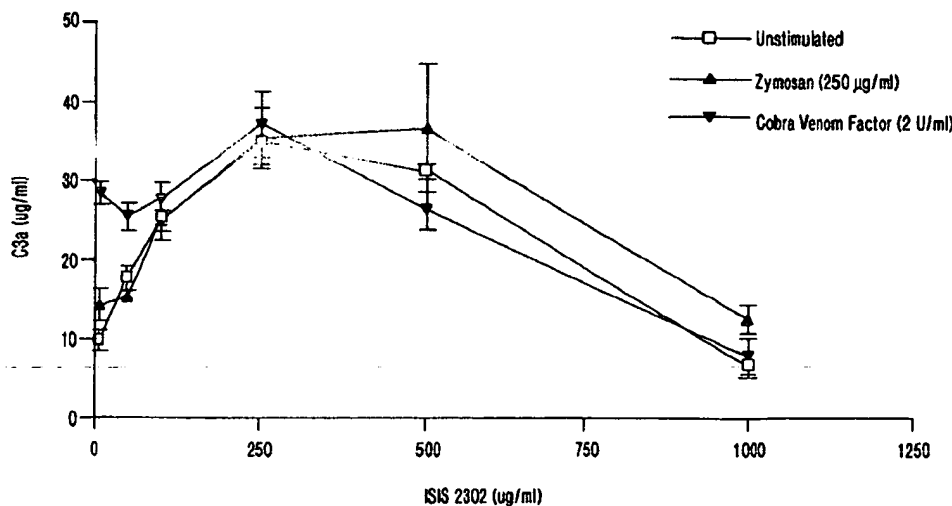
Henry, S.P. et al., Effects of Intravenous Infusion of Phosphorothioate Oligonucleotides on Coagulation, Complement Activation and Hemodynamics, Nucleosides & Nucleotides

(57) ABSTRACT

Oligomeric compounds are described wherein said compounds comprise modified oligonucleotides (P=S) which modulate complement activity. Methods and processes for the uses of such oligomeric compounds are also described. The oligomeric compounds may be used therapeutically to modulate complement activity in order to inhibit undesirable complement mediated events, such as for example, to treat inflammation, and/or to activate complement.

3 Claims, 5 Drawing Sheets

Inhibition of Complement Pathway in Monkey Serum



Complement Activation in Monkey Serum by
100 µg/ml ISIS 2302 Alternative Pathway Specific

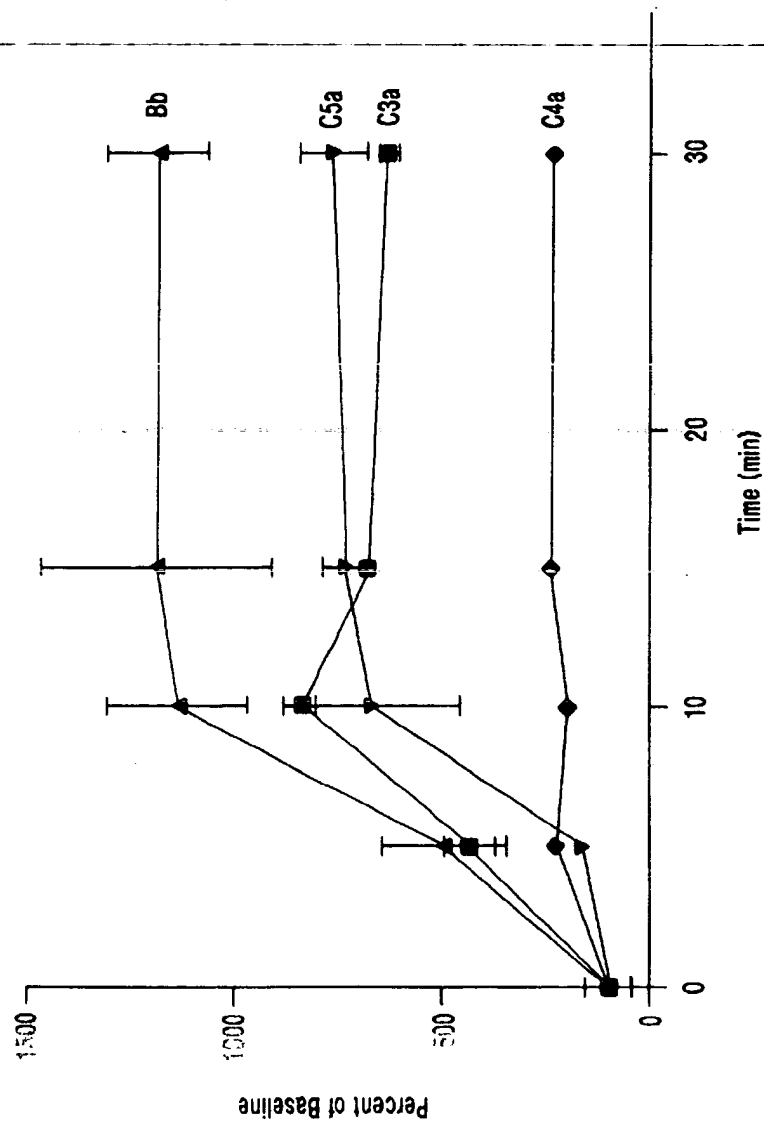


FIG. 1

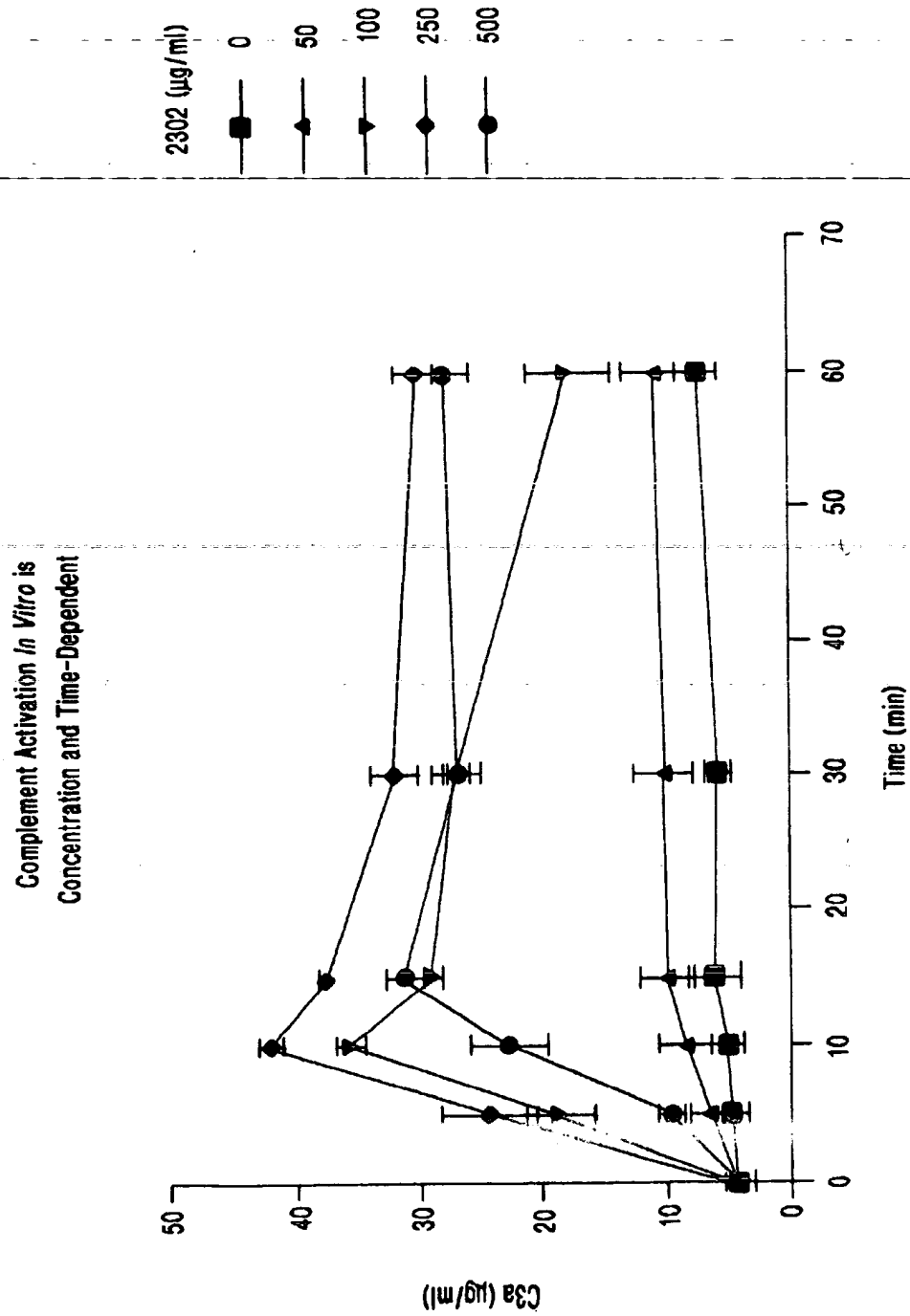


FIG. 2

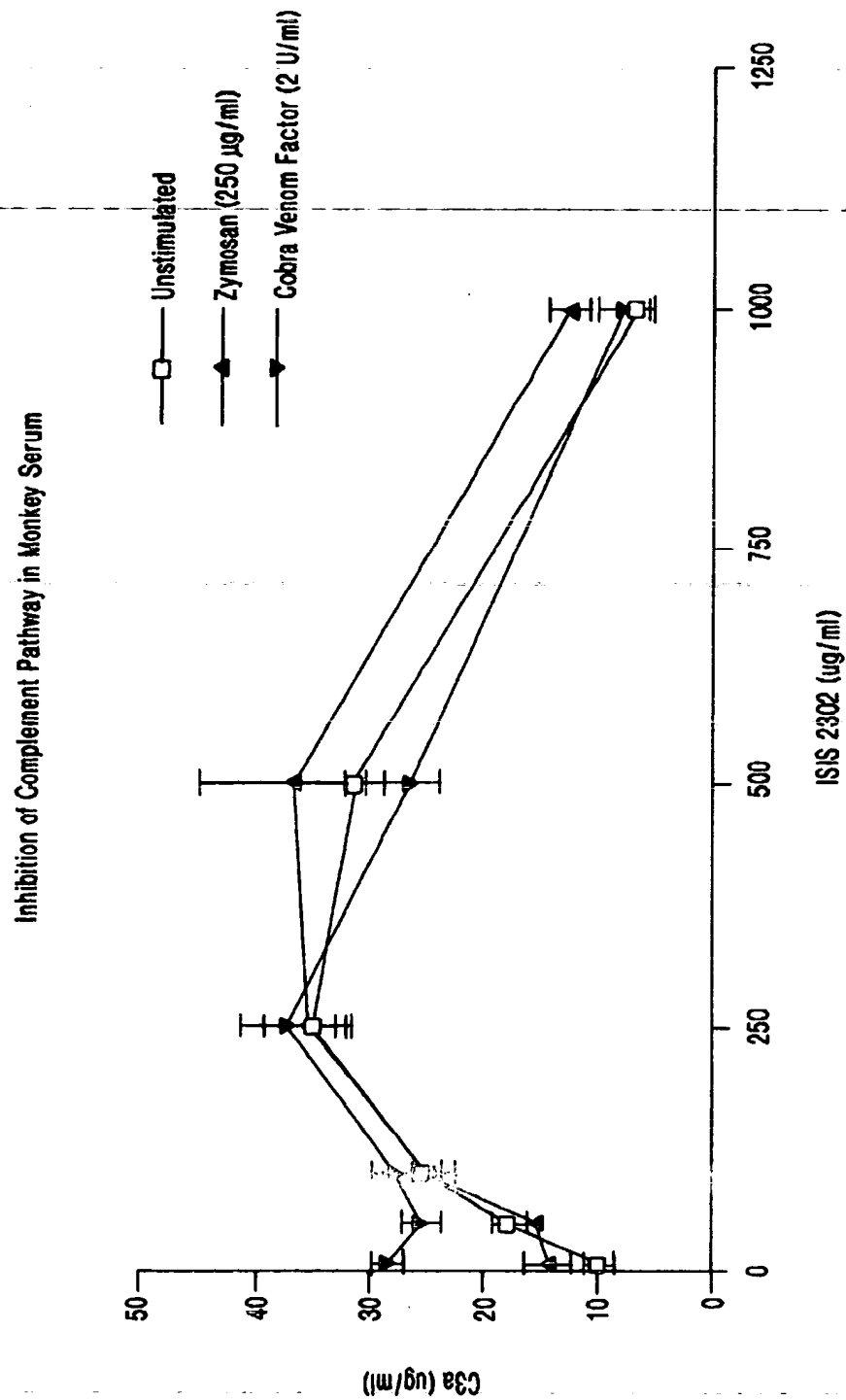


FIG. 3

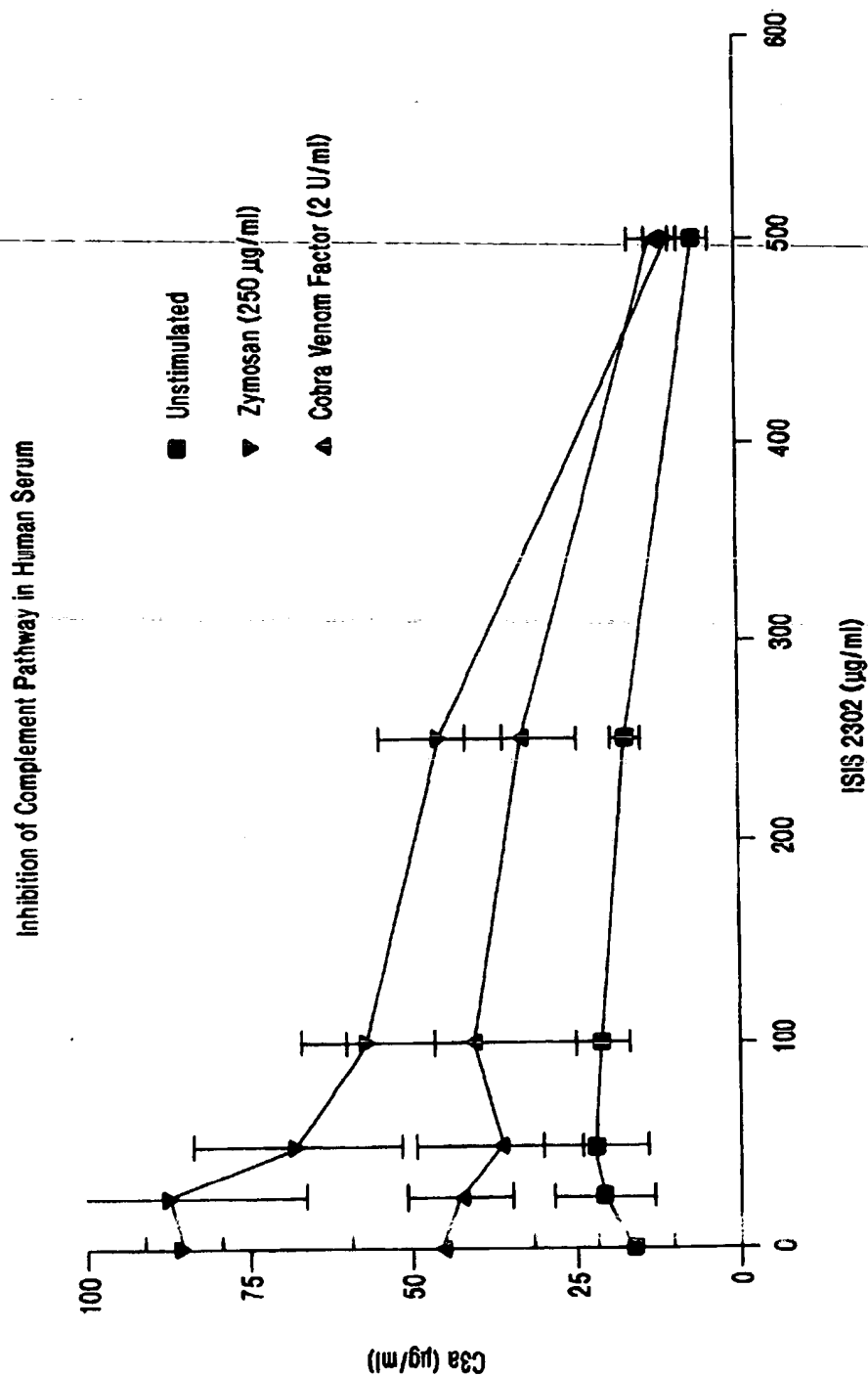


FIG. 4



1

INHIBITION OF COMPLEMENT ACTIVATION AND COMPLEMENT MODULATION BY USE OF MODIFIED OLIGONUCLEOTIDES

FIELD OF THE INVENTION

This invention concerns methods for the inhibition and/or modulation of the complement mediated immune response using synthetic nucleic acid molecules. The nucleic acid molecules may be synthetic nucleic acid molecules, such as oligonucleotides, wherein at least one of the ester linkage moieties of the oligonucleotide is replaced with a thioate linkage, such as in for example phosphorothioates. The methods described herein are useful as therapies for treating abnormal and/or undesirable conditions which can arise as a result of complement activation. Further uses as diagnostics and research reagents are also included in the present invention.

BACKGROUND OF THE INVENTION

The complement system is an important means by which a host defends itself against infection. The complement cascade system is a component of the immune system that helps provide a natural immunity against invading microbes and is also an effector arm of antibody mediated humoral immunity. Complement is responsible for activating cells and other molecules involved in the inflammatory process as well as being directly related to the destruction of microbial invaders. The activation of complement involves a cascade of proteolytic reactions that lead to the release of inflammatory mediators and result in the assembly of the microbial membrane attack complexes which, in turn, lyse invading microbial cells. This cascade system has been characterized as containing at least thirty serum and membrane proteins that are activated by antibody-antigen complexes or by the invasion, in a host or experimentally in culture, by a microorganism, or other antigenic molecules. Complement proteins may be grouped into three general categories: activating components, receptors, and positive and negative regulators.

The complement cascade consists of two major branches, the classical and alternative pathways. Though these pathways are initiated differently, they converge at the step of complement protein C3 activation (see FIG. 5). The complement cascade can mediate undesirable cellular damage in inflammatory, immune or autoimmune (auto-antibody-mediated) conditions such as; myasthenia gravis, immune complex excess syndromes such as systemic lupus erythematosus, ischemia-reperfusion states, hyper-acute rejection of transplants, organ failure conditions such as adult respiratory distress syndrome, Alzheimer's disease and related neurodegenerative disorders, among others.

A series of regulatory proteins are involved in the control of the complement cascade. These proteins are considered part of the complement system and act to block endogenous complement activity at either initiation or the formation of the membrane attack complex. Various therapeutic agents are being developed that block different steps in the complement cascade.

Complement is a group of serum proenzymes that are activated by antigen bound immunoglobulin or by membrane components on gram negative bacteria or fungi. The alternative pathway of the complement system is initialized by either the introduction of an endotoxin such as lipopolysaccharide [LPS], a component of the cell walls of gram negative bacteria or for instance by zymosan, a component of yeast cell walls, or by aggregated IgA.

The classical pathway of the complement system is initialized by Complement protein C1 binding to antigen bound

2

IgG or IgM. Both pathways converge at the formation of C3 convertase at which point an amplification takes place that generates literally thousands of C3a and C3b fragments. C3b fragments can bind to complement protein complex C4b2a to form C4b2a C3b which is called C5 convertase and generates thousands of C5a and C5b fragments. C3b can also be used to regenerate C3 convertase which causes a greater amplification of complement protein split product C3a. Split products C3a and C5a interact with receptors on mast cells to cause them to release histamine. Histamine induces inflammation which is generally considered protective, but in conditions characterized by improper complement activation and/or regulation inflammation can lead to damaged tissue.

One approach to inhibit complement mediated effects is by depleting complement. Depleting complement involves reducing the proteins responsible for the regeneration of C3 or C5 convertase and thereby reducing the amount of C3a and C5a produced. In this way complement is depleted or "used up". One such method for depleting complement component C3 convertase involves allowing C3 convertase to form and then binding split product C3b in order to reduce the further amplification of C3 convertase formation which can lead to C5 convertase formation.

In another approach for inhibiting complement the pathway is inhibited before the formation of C3 convertase. Inhibition of the formation of C3 convertase limits the production of split products C3b and C3a and further limits the formation of C5 convertase. Using this approach complement activation is blocked rather than depleted.

Candinas et al., describe the activation and depletion of complement by using cobra venom factor in conjunction with a recombinant soluble complement receptor type 1 protein (sCR1), and the use of such molecules in treating hyperacute xenograft rejection. (Candinas, D. et al., *Transplantation* 1996 15;62(3):336-342) sCR1 is a recombinant protein that has been shown to inhibit both the classical and alternative pathways of complement and thereby limits the production of proinflammatory products such as the anaphylatoxins (complement proteins C3a, C4a and C5a). sCR1 has also been described by Moore, F D Jr., as the first protein useful to treat adverse clinical situations which are complement-dependent, and further describes potential uses for sCR1 to treat thermal injury, ARDS, septic shock, and ischemia/reperfusion injury events such as myocardial infarction after thrombolytic therapy. (Moore, F D Jr., *Adv Immunol* 1994 56:267-299) U.S. Pat. No. 5,856,297, Fearon et al., issued Jan. 5, 1999 claims pharmaceutical compositions comprising a CR1 protein in various modifications and describes CR1 and the recombinant forms of the protein as being useful in the diagnosis and treatment of disorders involving complement activity and inflammation.

Other proteins have been investigated for their usefulness in inhibiting or modulating complement. For instance, Human IgG has been used to blunt complement activation in a pig-to-primate cardiac xenotransplantation hyperacute rejection study. The study determined that Human IgG caused a dose-dependent decrease in deposition of complement protein iC3b and a decrease in formation of C3 convertase. Furthermore, the infusion of IgG was found to prevent hyperacute rejection of porcine hearts transplanted into the primates. Magee J. C. et al., *J. Clin Invest* 1995 96(5):2404-2412. U.S. Pat. No. 5,851,528, Ko et al., issued Dec. 22, 1998, U.S. Pat. No. 5,679,546, Ko et al., issued Oct. 21, 1997, and U.S. Pat. No. 5,627,264, Fodor et al., issued May 6, 1997, describe chimeric proteins useful in inhibiting complement activation and describe methods to treat adverse conditions related to complement mediated inflammation. Sims, et al., U.S. Pat. No. 5,550,508, issued Aug. 27, 1996, describes polypeptides which act to inhibit comple-

ment C5b-9 complex activity. The protein is an 18 kDa protein found on the surface of human erythrocytes and is described as being useful in treating immune disease states when administered in effective amounts.

Magee, J. C. et al., *J. Clin. Invest.* 1995 96(5):2404-12, investigated the use of immunoglobulin to prevent complement-mediated hyperacute rejection in swine-to-primate xenotransplantation. In the study human IgG was added to human serum and was found to cause a dose-dependent decrease in the deposition of iC3b, cytotoxicity, and heparin sulfate release when the serum was incubated with porcine endothelial cells. It appears as if the decrease was caused by a decrease in the formation of C3 convertase on the endothelial cells. Furthermore, infusion of purified human IgG into primates prevented hyperacute rejection of porcine hearts in a xenotransplantation. Magee et al., determined that such results support the use of IgG as a therapeutic agent in humoral-mediated disease conditions.

U.S. Pat. No. 4,374,831, Joseph et al., issued Feb. 22, 1983, U.S. Pat. No. 4,087,548, Lenhard et al., issued May 2, 1978, U.S. Pat. No. 4,021,545, Nair et al., issued May 3, 1977, and U.S. Pat. No. 3,998,957, Conrow et al., issued Dec. 21, 1976, all describe chemical molecules which are useful as inhibitors and/or modulators of complement. Joseph et al., describe Bis-(β -D-glucopyranosyl-1-oxo)-arylene sulfate derivatives and methods for modulating complement in a warm blooded animal using pharmaceutical compounds comprising such molecules. Lenhard et al., describe complement inhibitory compounds such as C-substituted trisulfonic acids, acid ureides, and oxalyl amides and methods for inhibiting the complement system in a warm blooded animal by administering complement inhibitory amounts of the compounds comprising such molecules. Nair et al., claim methods for inhibiting the complement system in a warm blooded animal by using compositions comprising Inulin poly(H-sulfate). Conrow et al., describe 1,1'-[ureylenebis(sulfo-p-phenylene)]bis{[sulfo-1H, 8H-indazolo{2,3,4-cde}benzotriazol-9-ium hydroxide], bis (inner salts), and tetra salts as useful complement inhibitors.

In WO 95/32719 (1995), Galbraith describes the use of phosphorothioate oligonucleotides for depleting complement. The approach described involves administering, to a primate, an oligonucleotide 2 to 50 nucleotides in length containing at least one phosphorothioate internucleotide linkage, thereby stimulating vasodilation, and reducing complement activity by depleting complement.

Lin et al., WO 97/42317, describe oligonucleotides (aptamers) having phosphorothioate and/or substituted phosphonate linkages that are 37-61 base pairs in length which bind complement protein C3b and may be used diagnostically in vivo or in vitro to detect C3b in a biological sample. The aptamers may be used therapeutically to inhibit undesirable C3b-mediated complement events such as inflammation.

There has been and continues to be a long-felt need for methods for the inhibition and/or modulation of the complement mediated immune response using modified oligonucleotide compounds that might incorporate modifications for improving characteristics such as compound stability and cellular uptake. Such methods would be useful for therapeutically and prophylactically, as well as for diagnostic reagents and research reagents including reagents for the study of both cellular and in vitro events.

SUMMARY OF THE INVENTION

This invention relates to methods for modulating complement activation. These methods incorporate using modified oligonucleotides capable of inhibiting complement activation and/or initiating complement activation, depending on

oligonucleotide concentration, and thereby provide a method for modulating complement. The methods are also useful therapeutically for the treatment of abnormal and/or undesirable conditions which can arise as a result of complement activation. Other uses for the methods presently described, such as for example as diagnostics and research reagents, are also included.

Thus in a first aspect, the present invention features methods for modulating complement activation by independently administering to tissue, cells, cell/tissue culture, a bodily fluid, or a biological sample, a modified oligonucleotide in two different concentrations. Preferably, the first administered concentration initiates complement activation and the second concentration inhibits complement activation, although it is within the scope of the invention to have the first administered concentration inhibit complement activation and the second administered concentration initiate complement activation. In an embodiment of the invention, the initiating concentration of modified oligonucleotide is no greater than 80 μ g/ml, and more preferably between 50 μ g/ml and 80 μ g/ml, and the inhibitory concentration of modified oligonucleotide is at least 200 μ g/ml, and more preferably between 250 μ g/ml and 300 μ g/ml.

Most preferably the inhibitory concentration is greater than the activating concentration. Within the scope of the invention are further concentrations determined through methods such as titration wherein concentration levels are determined based on the condition or extent to which complement modulation is desired.

In preferred embodiments the methods are performed in vitro or ex vivo and are preferably performed on a bodily fluid sample or a biological sample such as for example a mammalian blood or serum sample. More preferably the mammalian blood or serum sample is from a primate, and most preferably the sample is from a human. Within this embodiment the biological fluid sample includes samples of tissue or cells, wherein the sample also contains complement components.

In other embodiments the methods are performed in vivo in a mammal. Preferably the mammal is a primate and most preferably the primate is a human.

In an additional embodiment the present invention provides methods for treating a human subject determined to have an abnormal or undesirable condition associated with complement activation by administering a first and second concentration of an oligonucleotide compound which modulates complement activity. Preferably the compound is administered in a first initiating concentration and a second inhibitory concentration. The oligonucleotide preferably contains one or more phosphorothioate modifications. It is preferred that the modulating concentrations are similar to those discussed above for both the first and second administration.

In a further embodiment the methods described herein are performed ex vivo on a cell culture, tissue sample, bodily fluid or a biological sample taken from a human. Most preferably the methods are performed in vivo in a human subject having an abnormal or undesirable condition associated with complement activation as determined by a licensed physician.

In an additional aspect the methods described herein feature an oligonucleotide which contains at least one phosphorothioate (P=S) modification and which modulates complement activity by initiating complement activation at a first oligonucleotide concentration and inhibiting complement activation at a second oligonucleotide concentration. The inhibition and initiation concentrations of the modified oligonucleotide are independent and separate measurements and are not considered to be the total concentration of

oligonucleotide in a sample or host. What is most preferred is that the first (initiating) concentration of the modified oligonucleotide be lower than the second (inhibiting) concentration of the same oligonucleotide.

It is preferred that the concentration of oligonucleotide required to initiate complement activation be less than or equal to 80 $\mu\text{g/ml}$, and more preferably the concentration required to initiate complement activation is between 50 $\mu\text{g/ml}$ and 80 $\mu\text{g/ml}$. Within this same aspect of the invention, it is presently preferred that the concentration of oligonucleotide required to inhibit complement activation be at least 200 $\mu\text{g/ml}$; more preferably the concentration required to inhibit complement activation is between 250 $\mu\text{g/ml}$ and 300 $\mu\text{g/ml}$. In regard to the first and second concentrations of oligonucleotide the preferred embodiments are not considered limiting.

Included in the invention, are methods for modulating complement activation in a cell culture, tissue or a bodily fluid by administering a modified oligonucleotide compound which inhibits complement activation and which contains at least one phosphorothioate modification and is conjugated to a complement activation inhibitory molecule. In preferred embodiments the methods are performed in vitro or ex vivo and are preferably performed on a bodily fluid sample, cell culture or a biological sample such as for example a mammalian blood or serum sample. More preferably the mammalian blood or serum sample is from a primate, and most preferably the sample is from a human. Within this embodiment the biological fluid sample includes samples of tissue or cells, wherein the sample also contains complement components.

Preferably, the modified oligonucleotide contains at least one phosphorothioate modification and is conjugated to a complement activation inhibitory molecule. More preferably the complement activation inhibitory molecule is a serum, vascular or cellular ligand, small complement binding molecule, or a complement specific ligand. Most preferably the complement activation inhibitory molecule binds complement Factor H. Preferably, the modified oligonucleotide to which the complement activation inhibitory molecule is bound is up to 60 oligonucleotides in length; in more preferred embodiments the modified oligonucleotide which inhibits complement activation is between 8 and 30 nucleotides in length. In an additional aspect, the invention features a modified oligonucleotide compound which inhibits complement activation.

In other embodiments the methods are performed in vivo in a mammal. Preferably the mammal is a primate and most preferably the primate is a human.

The term "independently administering" as used herein means providing one concentration (inhibitory or initiating) of modified oligonucleotide to the host and/or host cells at a time in order to modulate complement activity. The manner in which the modified oligonucleotide is administered may be adapted from, but is not limited to, intravenous infusion, needle injection, topical, needle-free injection as in, for example, an injection using a device like the Medijector™, and by aliquots using a pipette or similar device.

By use of term "culture" is meant the propagation of cells. Various culture methods exist and are included within the scope of the invention, methods such as, but not limited to, tissue culture methods, batch culture methods, enrichment culture methods, and ex vivo culture methods. In all culture methods the cells to be propagated should be in a nutritive environment which allows for continued cell growth, complement activation and/or inactivation. Tissue and cell culture methods are well understood in the art as these methods have been regularly practiced in various scientific fields for years. Such cultures may be propagated in natural serum or in artificial serum as described for example in U.S.

Pat. No. 4,657,866 to Kumar, Sudhir. Inasmuch as a culture represents a group of cells being observed for effects relating to complement activation or inhibition, included within the scope of the invention are cultures of cells on or in a host, such as a mass of burned tissue or cells, or a tumor growth, which must remain on or in the host to be propagated.

By the phrase "monitoring complement activity" is meant measuring products of the proteolytic complement cascade. Such products to be measured include, but are not limited to, complement proteins: C5a, C3a, and C4a. Methods for measuring products of the complement cascade are disclosed hereinbelow and can include antibody specific labeling of complement proteins C5A, C3a and C4a and performing ELISA assays to determine the relative concentration of the split products formed. In general monitoring of complement activity is performed on a biological sample that has been taken from a subject, patient or host, such as for example a serum or blood sample or other bodily fluid.

Further aspects of the invention are described within the description of the preferred embodiments. The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the invention and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings will herein briefly be described.

FIG. 1 is a line graph which shows the activation of complement in monkey serum by the addition of 100 $\mu\text{g/ml}$ of a phosphorothioate oligonucleotide (ISIS 2302; SEQ ID NO:1) of the invention as measured by the amounts of complement components compared to baseline levels.

FIG. 2 is a line graph which shows the measurement of complement component C3a to determine complement activation over a range of added concentrations of a phosphorothioate oligonucleotide of the present invention (ISIS 2302; SEQ ID NO:1).

FIG. 3 is a line graph which shows the inhibition of complement activation as measured by the amount of complement component C3a in monkey serum after stimulating complement activation with cobra venom factor or zymosan. Inhibition is measured over a concentration range of added phosphorothioate oligonucleotide (ISIS 2302; SEQ ID NO:1) as described herein.

FIG. 4 is a line graph which shows the inhibition of complement activation as measured by the amount of complement component C3a in human serum after stimulating complement activation with cobra venom factor or zymosan. Inhibition is measured over a concentration range of added phosphorothioate oligonucleotide (ISIS 2302; SEQ ID NO:1) as described herein.

FIG. 5 shows a schematic representation of the complement system.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Complement comprises a proteolytic cascade of serum and membrane proteins which are part of the immune system and are responsible for protecting hosts against foreign pathogens.

The complement system has powerful cytolytic activity which can damage an individual's own cells and should therefore be a target for modulation in order to reduce injury in various autoimmune events. In most cases individuals possess proteins which can control the extent of complement activation in serum or on the surfaces of "self" cells. Most of the proteins which inhibit complement activation in

serum serve to limit the generation of complement fragments such as C4b and C3b. Proteins such as C1-inhibitor, C4-binding protein, Factor H, and Factor I serves as normal inhibitors of complement in "normal" individuals. In a non-limiting example, C1 inhibitor is not present due to a genetic deletion or point mutations that produce an inactive form and results in hereditary angioedema of which there is also an acquired form usually due to auto-antibody to C1 INH. In such an abnormal condition such as angioedema as in others there is a need to help modulate complement in order to reduce the damage that can occur.

As described above, various methods have been investigated to inhibit complement activation. Willemin et al., *J. Immunol.* 15;159(4):1953-60, (1997), describes the use of glycosaminoglycans, such as heparin, to inhibit the interaction of complement component C1q with other complement activators and the assembly of the classical and alternative pathway C3 convertases. Naturally occurring glycosaminoglycans such as dextran sulfates, heparin, N-acetylheparin, heparan sulfate, dermatan sulfate, and chondroitin sulfates A and C were studied to determine their effectiveness at inhibiting the deposition of C4 and C3 on immobilized aggregated human IgG and to reduce fluid phase formation of C4b/c and C3b/c. In the study it was concluded that glycosaminoglycans such as the low molecular weight dextran sulfate (m.w. 5000) may serve as candidates for pharmacological manipulation of complement activation via potentiation of C1 inhibitor.

We have found that activation of the alternative pathway of complement occurs following the intravenous infusion of modified oligodeoxynucleotides (P=S oligo). By using monkey serum and whole blood we determined that modified oligonucleotides cause an increase in complement products Bb, C3a, and C5a. The concentration that was found to activate complement in these experimental systems was found to be up to roughly 50 $\mu\text{g}/\text{ml}$. By using the same modified oligonucleotide (ISIS 2302; SEQ ID NO:1) it was determined that at concentrations of roughly 250 $\mu\text{g}/\text{ml}$ and greater complement activation was inhibited in both the classical and alternative pathways as indicated by a reduction in complement components Bb, C3a, and C5a.

The present invention provides methods for modulating complement activity using modified oligonucleotides. The invention provides methods for using modified oligonucleotides which involve administering the oligonucleotides at one concentration to initiate complement activation and at another concentration to inhibit complement activation. The oligonucleotides of the present invention are modified to have improved pharmacokinetic properties. The methods described herein are useful as therapeutics for the treatment, prevention or diagnosis of abnormal and/or undesirable conditions which can arise as a result of complement mediated inflammatory effects.

By "abnormal and/or undesirable conditions" is meant any condition that have an inflammatory, immune or autoimmune component associated with the activation of the complement cascade. An abnormal and/or undesirable condition can be, but is not limited to: myasthenia gravis, immune complex excess syndromes such as systemic lupus, erythematousus, ischemia-reperfusion states, angioedema, hyper-acute rejection of transplants, organ failure conditions such as adult respiratory distress syndrome, Alzheimer's disease and related neurodegenerative disorders. Such conditions are generally determined by registered physicians.

Other envisioned treatments are for conditions in which a host is invaded by a foreign body which avoids the complement system and which may be targeted by an oligonucleotide according to the present invention in order to activate the complement system and eliminate the invading molecule.

Modifications of Oligonucleotides

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such "modified" or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target, increased stability in the presence of nucleases and an increase in bioavailability. In the present invention, oligonucleotides having at least one phosphorothioate modification are preferred.

Within the concepts of "oligonucleotides" and "modified" oligonucleotides, the present invention also includes compositions employing oligonucleotide compounds which are chimeric compounds. "Chimeric" oligonucleotide compounds or "chimeras," in the context of this invention, are nucleic acid compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or consist of an oligomeric sequence known to modify complement activation. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate oligodeoxynucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. RNase H-mediated target cleavage is distinct from the use of ribozymes to cleave nucleic acids.

By way of example, such "chimeras" may be "gapmers," i.e., oligonucleotides in which a central portion (the "gap") of the oligonucleotide serves as a substrate for, e.g., RNase H, and the 5' and 3' portions (the "wings") are modified in such a fashion so as to have greater affinity for, or stability when duplexed with, the target RNA molecule but are unable to support nuclease activity (e.g., 2'-fluoro- or 2'-methoxyethoxy-substituted). Other chimeras include "hemimers," that is, oligonucleotides in which the 5' portion of the oligonucleotide serves as a substrate for, e.g., RNase H, whereas the 3' portion is modified in such a fashion so as to have greater affinity for, or stability when duplexed with, the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-methoxyethoxy-substituted), or vice-versa.

A number of chemical modifications to oligonucleotides that confer greater oligonucleotide:RNA duplex stability have been described by Freier et al. (*Nucl. Acids Res.*, 1997, 25, 4429). Such modifications are preferred for the RNase H-refractory portions of chimeric oligonucleotides and may generally be used to enhance the affinity of an antisense compound for a target RNA. In this way, in a preferred embodiment, a chimeric molecule comprised of a modified oligonucleotide which modulates complement and an antisense portion may be administered in order to target a specific RNA molecule and modulate complement mediated adverse effects.

Chimeric modified oligonucleotide compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above, ligand-oligonucleotide constructs, or complement protein-oligonucleotide constructs as described herein. Some of these compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of some of these hybrid structures include, but are not limited to, U.S. Pat. Nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and U.S. Pat. No. 5,700,922, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned and allowed U.S. patent application Ser. No. 08/465,880, filed on Jun. 6, 1995, now U.S. Pat. No. 5,955,589 also herein incorporated by reference.

Modifications to an oligonucleotide molecule can alter the concentration of the molecule required to elicit the effect for which the molecule is designed. Non limiting examples include varying the amount of phosphorothioate linkages in the oligonucleotide or altering the oligonucleotide base composition and chemistry such as in the preparation of CpG oligodeoxynucleotides as described by Krieg et al., *Nature* 1995 374:546-549, Weiner et al., *Proc. Natl. Acad. Sci. USA* 1997 94:10833-10837, Liu, HM et al., *Blood* 1998 15:92(10):3730-3736, Boggs, RT et al., *Antisense Nucleic Acid Drug Dev* 1997 7(5):461-471, and Kline et al., *J. Immunol* 1998 15:160(6):2555-2559, which are all hereby incorporated herein in their entirety including any figures and drawings.

The present invention also includes compositions employing oligonucleotides that are substantially chirally pure with regard to particular positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (Cook et al., U.S. Pat. No. 5,587,361) and those having substantially chirally pure (Sp or Rp) alkylphosphonate, phosphoramidate or phosphotriester linkages (Cook, U.S. Pat. Nos. 5,212,295 and 5,521,302).

Oligonucleotides may contain modifications of the backbone sugar and/or nucleobase, singly or in combination. Specific examples of some preferred backbone modified oligonucleotides envisioned for this invention include those containing phosphorothioates (P=S oligonucleotides), phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

Other preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleotides with heteroatom backbones, and in particular —CH₂—NH—O—CH₂—, —CH₂—N(CH₃)—O—CH₂— [known as a methylene methylimino or MMI backbone], —CH₂—O—N(CH₃)—CH₂—, —CH₂—N(CH₃)—N(CH₃)—CH₂— and —O—N(CH₃)—CH₂—CH₂— [wherein the native phosphodiester backbone is represented as —O—P—O—CH₂—] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O—, S—, or N-alkyl; O—, S—, or N-alkenyl; O—, S—, or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl

and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_mCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O—CH₂CH₂OCH₃, also known as 2'-O—(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486–504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a $O(CH_2)_2ON(CH_2)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethyl (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O—CH₂—O—CH₂—N(CH₂)₂, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O—CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 5-azauracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858–859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, *Antisense Research and Applications*, pages 289–302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Certain of these

nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6–1.2° C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276–278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and U.S. Pat. No. 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553–6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053–1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306–309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765–2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533–538), an aliphatic chain, e.g., dodecanol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111–1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327–330; Svinarchuk et al., *Biochimie*, 1993, 75, 49–54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651–3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777–3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969–973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651–3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229–237), or an octadecylamine or hexylamino-carbonyl-cholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923–937).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,108; 5,319,104; 5,511,213; 5,512,730; 5,521,118; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,582,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the

aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide.

Another preferred additional or alternative modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide. Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2' position of the sugar of any nucleotide. The N⁶ position of a purine nucleobase may also be utilized to link a lipophilic moiety to an oligonucleotide of the invention (Gebeyehu, G., et al., *Nucleic Acids Res.*, 1987, 15, 4513). Such lipophilic moieties include but are not limited to a cholesteryl moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 111; Kabanov et al., *FEBS Lett.*, 1990, 259, 327; Svinarchuk et al., *Biochimie*, 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides, as disclosed in U.S. Pat. Nos. 5,138,045, 5,218,105 and 5,459,255, the contents of which are hereby incorporated by reference in their entirety.

In other preferred embodiments the compound may be a ligand conjugated oligomeric compound having improved pharmacokinetic properties. Such oligomeric compounds are prepared having covalently attached ligands or proteins that bind reversibly to or interact with one or more serum, vascular or cellular proteins. This reversible binding is expected to decrease urinary excretion, increase serum half life and greatly increase the distribution of oligomeric compounds thus conjugated. In the case of binding a complement protein, such as for example complement factor H or a ligand thereof, in the context of the present invention the binding is to further inhibit complement activity. The binding of particular drugs to plasma protein has been previously shown to enhance the disposition and efficacy of drugs (Herve et al., *Clin. Pharmacokin.*, 1994, 26:44).

An oligomeric agent should be able to overcome inherent factors such as rapid degradation in serum, short half life in serum and rapid filtration by the kidneys with subsequent excretion in the urine. Oligonucleotides that overcome these inherent factors have increased serum half lives, distribution, cellular uptake and hence improved efficacy. These enhanced pharmacokinetic parameters have been shown for selected drug molecules that bind plasma proteins (Olson and Christ, *Annual Reports in Medicinal Chemistry*, 1996, 31:327). Two proteins that have been studied more than most are human serum albumin (HSA) and α -1-acid glycoprotein. HSA binds a variety of endogenous and exogenous ligands with association constants typically in the

range of 10^4 to 10^6 M⁻¹. Association constants for ligands with α -1-acid glycoprotein are similar to those for HSA.

At least for therapeutic purposes, oligonucleotides should have a degree of stability in serum to allow distribution and cellular uptake. The prolonged maintenance of therapeutic levels of oligomeric agents in serum will have a significant effect on the distribution and cellular uptake and unlike conjugate groups that target specific cell receptors the increased serum stability will affect all cells. Numerous efforts have focused on increasing the cellular uptake of oligonucleotides including increasing the membrane permeability via conjugates and cellular delivery of oligonucleotides.

Many drugs reversibly bind to plasma proteins. A representative list, which is not meant to be inclusive, includes: aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triodobenzoic acid, flufenamic acid, folic acid, benzothiadiazides, chlorothiazide, diazepam (such as for example fludiazepam and diazepam) indomethacin, barbiturates (such as for example quinalbarbitone), cephalosporins, sulfa drugs, antidiabetics (such as for example tolbutamide), antibacterials (such as for example a group of quinolones; nalidixic acid and cinoxacin) and several antibiotics. Serum albumin is the most important protein among all plasma proteins for drug binding, although binding to other proteins (for example, macroglobulin G₂, immunoglobulins, lipoproteins, alpha-1-acid glycoprotein, thrombin) is also important.

Ligands that bind serum, vascular or cellular proteins may be attached via an optional linking moiety to one or more sites on an oligonucleotide of the invention. These sites include one or more of, but are not limited to, the 2' position, 3'-position, 5'-position, the internucleotide linkage, and a nucleobase atom of any nucleotide residue. The attachment of ligands to such structures can be performed, according to some preferred embodiments of the invention, using a linking group, or without the use of such a linking group. Preferred linking groups of the invention include, but are not limited to, 6-aminoalkoxy linkers, 6-aminoalkylamino linkers, cysteamine, heterobifunctional linkers, homobifunctional linkers, and a universal linker (derived from 3-dimethoxytrityloxy-2-aminopropanol). A particularly preferred linking group for the synthesis of ligand conjugated oligonucleotides of the invention is a 6-aminohexyloxy group. A variety of heterobifunctional and homobifunctional linking moieties are available from Pierce Co. (Rockford, Ill.). Such heterobifunctional and homobifunctional linking moieties are particularly useful in conjunction with the 6-aminoalkoxy and 6-aminoalkylamino moieties to form extended linkers useful for linking ligands to a nucleoside. Further useful linking groups that are commercially available are 5'-Amino-Modifier C6 and 3'-Amino-Modifier reagents, both available from Glen Research Corporation (Sterling, Va.). 5'-Amino-Modifier C6 is also available from Aid (Applied Bio-systems, Inc., Foster City, Calif.) as Aminolink-2, while the 3'-Amino-Modifier is also available from Clontech Laboratories Inc. (Palo Alto, Calif.). In addition, a nucleotide analog bearing a linking group pre-attached to the nucleoside is commercially available from Glen Research Corporation under the tradename "Amino-Modifier-dT." This nucleoside-linking group reagent, a uridine derivative having an [N(7-trifluoroacetylaminohexyl) 3-acrylamido] substituent group at the 5 position of the pyrimidine ring, is synthesized as per the procedure of Jablonski et al. (*Nucleic Acid Research*, 1986, 14:6115). The present invention also includes as nucleoside analogs adenine nucleosides functionalized to include a linker on the N6 purine amino group, guanine nucleosides functionalized to include a linker at the exocyclic N2 purine amino group, and cytosine nucleosides functionalized to include a linker

15

on either the N4 pyrimidine amino group or the 5 pyrimidine position. Such nucleoside analogs are incorporated into oligonucleotides with a ligand attached to the linker either pre- or post-oligomerization.

In a preferred embodiment of the present invention ligand molecules are selected for conjugation to oligonucleotides on the basis of their affinity for one or more complement proteins. These proteins may be serum, vascular or cellular proteins. Serum proteins are proteins that are present in the fluid portion of the blood, obtained after coagulation and removal of the fibrin clot and blood cells, as distinguished from the plasma in circulating blood. Vascular proteins are proteins that are present in portions of the vascular system relating to or containing blood vessels. Cellular proteins are membrane-proteins which have at least a portion of the protein extending extracellularly and assisting in the process of endocytosis.

Many ligands having an affinity for proteins are well documented in the literature and are amenable to use in the present invention. A preferred group of ligands are small molecules including drug moieties. According to the present invention, drug moieties include, but are not limited to, warfarin and coumarins including substituted coumarins, isocoumarin derivatives, 7-anilinocoumarin-4-acetic acid, profens including ibuprofen, enantiomers of ibuprofen (r-ibuprofen and s-ibuprofen), ibuprofen analogs, ketoprofen, carprofen, etodolac, suprofen, indoprofen, fenbufen, arylpropionic acids, arylalkanoic acids, 2-aryl-2-fluoropropionic acids, glibenclamide, acetohexamide, arylalkanoic acids, tolbutamide, glitazide, metformin, curcumin, digitoxin, digoxin, diazepam, benzothiadiazides, chlorothiazide, diazepines, benzodiazepines, naproxen, phenyl butazone, oxyphenbutazone, dansyl amide, dansylsarcosine, 2,3,5-triiodobenzoic acid, palmitic acid, aspirin, salicylates, substituted salicylates, penicillin, flurbiprofen, piroprofen, oxaprozin, flufenamic acid, deoxycholic acid, glycyrrhizin, azathioprine, butibufen, ibufenac, 5-fluoro-1-typtaphan, 5-fluoro-salicylic, acidazapropanazone, mefenamic acid, indomethacin, flufenamic acid, bilirubin, ibuprofen, lysine complexes, diphenyl hydantoin, valproic acid, tolmetin, barbiturates (such as, for example, quinalbarbitone), cephalosporins, sulfa drugs, antidiabetics (such as, for example, tolbutamide), antibacterials (such as, for example, quinolones, nalidixic acid and cinoxacin) and several antibiotics.

In one embodiment of the present invention the drug moiety bears a carboxylic acid group. In another embodiment of the present invention the drug moiety is a propionic acid derivative.

In one preferred embodiment of the invention the protein for binding a ligand conjugated oligomeric compound is a serum protein. It is preferred that the serum protein bound by a conjugated oligomeric compound is an immunoglobulin (an antibody). Preferred immunoglobulins are immunoglobulin G and immunoglobulin M. Immunoglobulins are known to appear in blood serum and tissues of vertebrate animals. A more preferred protein for binding to a ligand conjugated oligomer is albumin.

In another embodiment of the invention the serum protein for binding by a conjugated oligomeric compound is a lipoprotein. Lipoproteins are blood proteins having molecular weights generally above 20,000 that carry lipids and are recognized by specific cell surface receptors. The association with lipoproteins in the serum will initially increase pharmacokinetic parameters such as half life and distribution. A secondary consideration is the ability of lipoproteins to enhance cellular uptake via receptor-mediated endocytosis.

In yet another embodiment the serum protein for binding by a ligand conjugated oligomeric compound is α -2-macroglobulin. In yet a further embodiment the serum

16

protein targeted by a ligand conjugated oligomeric compound is α -1-glycoprotein.

As used herein, the term "protected" means that the indicated moiety has a protecting group appended thereon. In some preferred embodiments of the invention compounds contain one or more protecting groups. A wide variety of protecting groups can be employed in the methods of the invention. In general, protecting groups render chemical functionalities inert to specific reaction conditions, and can be appended to and removed from such functionalities in a molecule without substantially damaging the remainder of the molecule.

Representative hydroxyl protecting groups, for example, are disclosed by Beaucage et al. (*Tetrahedron*, 1992, 48:2223-2311). Further hydroxyl-protecting groups, as well as other representative protecting groups, are disclosed in Greene and Wuts, *Protective Groups in Organic Synthesis*, Chapter 2, 2d ed., John Wiley & Sons, New York, 1991, and *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y., 1991, each of which is hereby incorporated by reference in its entirety.

Examples of hydroxyl protecting groups include, but are not limited to, t-butyl, t-butoxymethyl, methoxymethyl, tetrahydropyranyl, 1-cetoxyethyl, 1-(2-chloroethoxy)ethyl, 2-trimethylsilylethyl, p-chlorophenyl, 2,4-dinitrophenyl, benzyl, 2, 6-dichlorobenzyl, diphenylmethyl, p,p'-dinitrobenzhydryl, p-nitrobenzyl, triphenylmethyl, trimethylsilyl, triethylsilyl, t-butyl dimethylsilyl, t-butyl diphenylsilyl, triphenylsilyl, benzoylformate, acetate, chloroacetate, trichloroacetate, trifluoroacetate, pivaloate, benzoate, p-phenylbenzoate, 9-fluorenylmethyl carbonate, mesylate and tosylate.

Amino-protecting groups stable to acid treatment are selectively removed with base treatment, and are used to make reactive amino groups selectively available for substitution. Examples of such groups are the Fmoc (E. Atherton and R. C. Sheppard in *The Peptides*, S. Udenfriend, J. Meienhofer, Eds., Academic Press, Orlando, 1987, volume 9, p.1) and various substituted sulfonyl ethyl carbamates exemplified by the Nsc group (Samukov et al., *Tetrahedron Lett*, 1994, 35:7821; Verhart and Tesser, *Rec. Trav. Chim. Pays-Bas*, 1987, 107:621).

Additional amino-protecting groups include, but are not limited to, carbamate-protecting groups, such as 2-trimethylsilylethoxycarbonyl (Teoc), 1-methyl-1-(4-biphenyl)ethoxycarbonyl (Bpoc), t-butoxycarbonyl (BOC), allyloxycarbonyl (Alloc), 9-fluorenylmethyloxycarbonyl (Fmoc), and benzyloxycarbonyl (Cbz); amide-protecting groups, such as formyl, acetyl, trihaloacetyl, benzoyl, and nitrophenylacetyl; sulfonamide-protecting groups, such as 2-nitrobenzenesulfonyl; and imine- and cyclic imide-protecting groups, such as phthalimido and dithiasuccinoyl. Equivalents of these aminoprotecting groups are also encompassed by the compounds and methods of the present invention.

In a preferred embodiment of the present invention oligonucleotides are provided including a number of linked nucleosides wherein at least one of the nucleosides is a 2'-functionalized nucleoside having a ligand molecule linked to the 2'-position of the nucleoside; a heterocyclic base functionalized nucleoside having a ligand molecule linked to the heterocyclic base of the nucleoside, a 5' terminal nucleoside having a ligand molecule linked to the 5'-position of the nucleoside, a 3' terminal nucleoside having a ligand molecule linked to the 3'-position of the nucleoside, or an inter-strand nucleoside having a ligand molecule linked to an inter-strand linkage linking said inter-strand nucleoside to an adjacent nucleoside.

Ligand conjugated oligonucleotides may be synthesized by the use of an oligonucleotide that bears a pendant reactive

functionality such as that derived from the attachment of a linking molecule onto the oligonucleotide. This reactive oligonucleotide may be reacted directly with commercially available ligands, ligands that are synthesized bearing a variety of protecting groups, or ligands that have a linking moiety attached thereto. The methods of the present invention facilitate the synthesis of ligand conjugated oligonucleotides by the use of, in some preferred embodiments, nucleoside monomers that have been appropriately conjugated with ligands and that may further be attached to a solid support material. Such ligand-nucleoside conjugates optionally attached to a solid support material are prepared according to some preferred embodiments of the methods of the present invention via reaction of a selected serum binding ligand with a linking moiety located on a 2', 3', or 5' position of a nucleoside or oligonucleotide.

The above described conjugation of ligands to oligomeric compounds has been shown to increase the concentration of such compounds in serum. According to such methods, a drug moiety that is known to bind to a serum protein is selected and conjugated to an oligonucleotide, thus forming a conjugated oligonucleotide. This conjugated oligonucleotide is then added to the serum.

Conjugation of a ligand also provides a way to increase the capacity of serum for an oligonucleotide. According to such methods, a drug moiety that is known to bind to a serum protein is selected and conjugated to an oligonucleotide, thus forming a conjugated oligonucleotide. This conjugated oligonucleotide is then added to the serum.

Ligand conjugation can also increase the binding of an oligonucleotide to a portion of the vascular system. According to such methods, a drug moiety that is known to bind to a vascular protein is selected. The vascular protein selected is a protein which resides, in part, in the circulating serum and, in part, in the non-circulating portion of the vascular system. This drug moiety is conjugated to an oligonucleotide to form a conjugated oligonucleotide, which is then added to the vascular system.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., *Helv. Chim. Acta*, 1995, 78, 486-504). It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling Va.) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

Complement Modulation

The modified oligonucleotide compounds of the present invention include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and prodrugs of such nucleic acids.

"Pharmaceutically acceptable salts" are physiologically and pharmaceutically acceptable salts of the nucleic acids of the invention: i.e., salts that retain the desired biological

activity of the parent compound and do not impart undesired toxicological effects thereto (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma. Sci.*, 1977, 66:1, which is incorporated herein by reference in its entirety).

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE ((S-acetyl-2-thioethyl) phosphate) derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published Dec. 9, 1993, which is incorporated herein by reference in its entirety.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may include pharmaceutically acceptable carriers, thickeners, diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1). One or more penetration enhancers from one or more of these broad categories may be included.

The compositions of the present invention may additionally contain other adjuvant components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to

enhance the in vivo stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layers made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.*, 1995, 6, 698). Liposomal modified oligonucleotide compositions are prepared according to the disclosure of co-pending U.S. patent application Ser. No. 08/961,469 to Hardee et al., filed Oct. 31, 1997, U.S. Pat. No. 6,083,923, incorporated herein by reference in its entirety.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal and transdermal), oral or parenteral, or by aliquots using a pipette or the like. Parenteral administration includes intravenous drip, injection or infusion, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration. Injection includes both needle injection and needle-free injection as in, for example, an injection using a device like the Medijector™. For oral administration, it has been found that oligonucleotides with at least one 2'-substituted ribonucleotide are particularly useful because of their absorption and distribution characteristics. U.S. Pat. No. 5,591,721 issued to Agrawal et al. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective in vitro and in vivo animal models. In general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it

may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 µg to 100 g per kg of body weight, once or more daily, to once every 20 years.

By "ex vivo" is meant removing a sample of blood, serum and/or bone marrow from a subject in need of complement modulation, treating the sample with the modified oligonucleotide described herein, and returning the sample to the subject.

Thus, in the context of this invention, by "therapeutically effective amount" is meant the amount of the compound which is required to have a therapeutic effect on the treated mammal. This amount, which will be apparent to the skilled artisan, will depend upon the type of mammal, the age and weight of the mammal, the type of disease to be treated, perhaps even the gender of the mammal, and other factors which are routinely taken into consideration when treating a mammal with a disease. A therapeutic effect is assessed in the mammal by measuring the effect of the compound on the disease state in the animal. For example, if the disease to be treated is an ischaemia-reperfusion event, a reduction in tissue damage is an indication that the administered dose has a therapeutic effect. In an example of a chimeric oligonucleotide usage, if the disease to be treated is psoriasis, a reduction or ablation of the skin plaque and a reduced activation of complement occurs this would also be an indication that the administered dose has a therapeutic effect. Similarly, in mammals being treated for cancer, therapeutic effects are assessed by measuring both the amount of complement activation and the rate of growth or the size of the tumor, or by measuring the production of compounds such as cytokines, production of which is an indication of the progress or regression of the tumor.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy Amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyl-diisopropyl phosphoramidites are purchased from commercial sources (e.g. ChemGenes, Needham Mass. or Glen Research, Inc. Sterling Va.).

Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Pat. No. 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides is utilized, except the wait step after pulse delivery of tetrazole and base is increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides are synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling Va. or ChemGenes, Needham Mass.).

2'-Fluoro Amidites

2'-Fluorodeoxyadenosine Amidites

2'-fluoro oligonucleotides may be synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and U.S. Pat. No. 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N⁶-benzoyl-2'-deoxy-2'-fluoroadenosine is synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus

21

N6-benzoyl-9-beta-D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-diisobutylhydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups is accomplished using standard methodologies and standard methods may be used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine is accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutryl-arabinofuranosylguanine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to give diisobutryl-di-THP-protected arabinofuranosylguanine. Selective O-deacylation and triflation is followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies may be used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures may be used to obtain the 5'-DMT and 5'-DMT-3' phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures may be used to obtain the 5'-DMT and 5'-DMT-3' phosphoramidites.

2'-O-(2-Methoxyethyl) Modified Amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) are added to DMF (300 mL). The mixture is heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution is concentrated under reduced pressure. The resulting syrup is poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether is decanted and the residue is dissolved in a minimum amount of methanol (ca. 400 mL). The solution is poured into fresh ether (2.5 L) to yield a stiff gum. The ether is decanted and the gum is dried in a vacuum oven (60° C. at 1 mm Hg for 24 h) to give a solid that is crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum is consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material is used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4° C.).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) may be added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160° C. After heating for 48 hours at 155-160° C., the vessel is opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue is suspended in hot acetone (1 L). The insoluble salts may be filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) is dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) is packed in CH₂Cl₂/acetone/MeOH (20:5:3)

22

containing 0.5% Et₃NH. The residue is dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product is eluted with the packing solvent to give 160 g (63%) of product. Additional material is obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) is co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) is added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) is added and the reaction stirred for an additional one hour. Methanol (170 mL) is then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent is evaporated and triturated with CH₃CN (200 mL). The residue is dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase is dried over Na₂SO₄, filtered and evaporated. 275 g of residue is obtained. The residue is purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions may be evaporated to give 164 g of product. Approximately 20 g additional is obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL) of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) may be combined and stirred at room temperature for 24 hours. The reaction is monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) is added and the mixture evaporated at 35° C. The residue is dissolved in CHCl₃ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers may be back extracted with 200 mL of CHCl₃. The combined organics may be dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue is purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane (4:1). Pure product fractions may be evaporated to yield 96 g (84%). An additional 1.5 g is recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution is prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) is added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5° C. and stirred for 0.5 h using an overhead stirrer. POCl₃ is added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10° C., and the resulting mixture stirred for an additional 2 hours. The first solution is added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture is stored overnight in a cold room. Salts may be filtered from the reaction mixture and the solution is evaporated. The residue is dissolved in EtOAc (1 L) and the insoluble solids may be removed by filtration. The filtrate is washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue is triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) is stirred at room temperature for 2 hours. The dioxane solution is evaporated and the residue azeotroped with MeOH (2x200 mL). The residue is dissolved in MeOH (300 mL) and

transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas is added and the vessel heated to 100°C . for 2 hours (TLC showed complete conversion). The vessel contents may be evaporated to dryness and the residue is dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics may be dried over sodium sulfate and the solvent is evaporated to give 85 g (95%) of the title compound. N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) is dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) is added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent is evaporated and the residue azeotroped with MeOH (200 mL). The residue is dissolved in CHCl_3 (700 mL) and extracted with saturated NaHCO_3 (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO_4 and evaporated to give a residue (96 g). The residue is chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions may be evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) is dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl) phosphite (40.5 mL, 0.123 M) may be added with stirring, under a nitrogen atmosphere. The resulting mixture is stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture is extracted with saturated NaHCO_3 (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes may be back-extracted with CH_2Cl_2 (300 mL), and the extracts may be combined, dried over MgSO_4 and concentrated. The residue obtained is chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions may be combined to give 90.6 g (87%) of the title compound. 2'-O-(Aminoxyethyl) nucleoside amidites and 2'-O-(dimethylaminoxyethyl) nucleoside amidites

2'-(Dimethylaminoxyethoxy) Nucleoside Amidites

2'-(Dimethylaminoxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminoxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine

O²-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0 g, 0.416 mmol), dimethylaminopyridine (0.66 g, 0.013 eq, 0.0054 mmol) may be dissolved in dry pyridine (300 mL) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8 g, 119.0 mL, 1.1 eq, 0.458 mmol) is added in one portion. The reaction is stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution is concentrated under reduced pressure to a thick oil. This is partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer is dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil is dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600 mL) and the solution is cooled to -10°C . The resulting crystalline product is collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C , 1 mm Hg, 24 h) to 149 g (74.8%) of white solid. TLC and NMR may be consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor is added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) is added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) may be added with manual stirring. The reactor is sealed and heated in an oil bath until an internal temperature of 160°C . is reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel is cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction is stopped, concentrated under reduced pressure (10 to 1 mm Hg) in a warm water bath (40 – 100°C .) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue is purified by column chromatography (2 kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions may be combined, stripped and dried to product as a white crisp foam (84 g, 50%), contaminated starting material (17.4 g) and pure reusable starting material 20 g. The yield based on starting material less pure recovered starting material is 58%. TLC and NMR may be consistent with 99% pure product.

2'-O-[(2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20 g, 36.98 mmol) is mixed with triphenyl phosphine (11.63 g, 44.36 mmol) and N-hydroxyphthalimide (7.24 g, 44.36 mmol). It is then dried over P_2O_5 under high vacuum for two days at 40°C . The reaction mixture is flushed with argon and dry THF (369.8 mL, Aldrich, sure seal bottle) is added to get a clear solution. Diethylazodicarboxylate (6.98 mL, 44.36 mmol) is added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition is complete, the reaction is stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethyl acetate:hexane, 60:40). The solvent is evaporated in vacuum. Residue obtained is placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-[(2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-[(2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (3.1 g, 4.5 mmol) is dissolved in dry CH_2Cl_2 (4.5 mL) and methylhydrazine (300 mL, 4.64 mmol) is added dropwise at ambient temperature under an argon atmosphere. The mixture is stirred for 16 h at ambient temperature. The mixture is filtered, the filtrate is washed with ice cold CH_2Cl_2 and the combined organic phase is washed with water, brine and dried over anhydrous Na_2SO_4 . The solution is concentrated to get 2'-O-(aminoxyethyl) thymidine, which is then dissolved in MeOH (67.5 mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) is added and the resulting mixture is stirred for 1 h. Solvent is removed under vacuum; residue chromatographed to get 5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77 g, 3.12 mmol) is dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPIS) in dry

25

MeOH (30.6 mL). Sodium cyanoborohydride (0.39 g, 6.13 mmol) is added to this solution at 10° C. under inert atmosphere. The reaction mixture is stirred for 10 minutes at 10° C. After that the reaction vessel is removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH₂Cl₂). Aqueous NaHCO₃ solution (5%, 10 mL) is added and extracted with ethyl acetate (2x20 mL). Ethyl acetate phase is dried over anhydrous Na₂SO₄, evaporated to dryness. Residue is dissolved in a solution of 1M PPTS in MeOH (30.6 mL). Formaldehyde (20% w/w, 30 mL, 3.37 mmol) is added and the reaction mixture is stirred at room temperature for 10 minutes. Reaction mixture cooled to 10° C. in an ice bath, sodium cyanoborohydride (0.39 g, 6.13 mmol) is added and reaction mixture stirred at 10° C. for 10 minutes. After 10 minutes, the reaction mixture is removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO₃ (25 mL) solution is added and extracted with ethyl acetate (2x25 mL). Ethyl acetate layer is dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue obtained is purified by flash column chromatography and eluted with 5% MeOH in CH₂Cl₂ to get 5'-O-tert-butylidiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6 g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91 mL, 24.0 mmol) is dissolved in dry THF and triethylamine (1.67 mL, 12 mmol, dry, kept over KOH). This mixture of triethylamine-2 HF is then added to 5'-O-tert-butylidiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40 g, 2.4 mmol) and stirred at room temperature for 24 hrs. Reaction is monitored by TLC (5% MeOH in CH₂Cl₂). Solvent is removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH₂Cl₂ to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766 mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750 mg, 2.17 mmol) is dried over P₂O₅ under high vacuum overnight at 40° C. It is then co-evaporated with anhydrous pyridine (20 mL). The residue obtained is dissolved in pyridine (11 mL) under argon atmosphere. 4-dimethylaminopyridine (26.5 mg, 2.60 mmol), 4,4'-dimethoxytrityl chloride (880 mg, 2.60 mmol) is added to the mixture and the reaction mixture is stirred at room temperature until all of the starting material disappeared. Pyridine is removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH₂Cl₂ (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.13 g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08 g, 1.67 mmol) is co-evaporated with toluene (10 mL). To the residue N,N-diisopropylphosphoramidite (0.29 g, 1.67 mmol) is added and dried over P₂O₅ under high vacuum overnight at 40° C. Then the reaction mixture is dissolved in anhydrous acetonitrile (8.4 mL) and 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphoramidite (2.12 mL, 6.08 mmol) is added. The reaction mixture is stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction is monitored by TLC (hexane:ethyl acetate 1:1). The solvent is evaporated, then the residue is dissolved in ethyl acetate (70 mL) and washed with 5% aqueous NaHCO₃ (40 mL). Ethyl acetate layer is dried over anhydrous Na₂SO₄ and concentrated. Residue obtained is chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04 g, 74.9%).

26

2'-(Aminooxyethoxy) nucleoside amidites 2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly. N2-isobutryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl)diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinasso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) Nucleoside Amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl Uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O2-2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155 C. for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:9 (v/v) as eluent. The column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2 (2-N,N-dimethylaminoethoxy)ethyl]-5-methyl Uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH₂Cl₂ (2x200 mL). The combined CH₂Cl₂ layers are washed with saturated NaHCO₃ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using

27

MeOH:CH₂Cl₂:Et₃N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH₂Cl₂ (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide Synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle is replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step is increased to 68 sec and is followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55° C. (18 h), the oligonucleotides may be purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Pat. No. 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Pat. No. 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Pat. Nos. 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Pat. No. 5,256,775 or U.S. Pat. No. 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Pat. No. 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Pat. No. 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Pat. Nos. 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds

28

having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Pat. Nos. 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Pat. Nos. 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Pat. No. 5,223,618, herein incorporated by reference.

Example 4

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]-[2'-O-deoxy]-[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-O-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample is again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]-[2'-deoxy]-[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]-[2'-deoxy]-[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides may be prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]-[2'-deoxy Phosphorothioate]-[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl) phosphodiester]-[2'-deoxy phosphorothioate]-[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidation with iodine to generate the phosphodiester internucleotide linkages within the wing

29

portions of the chimeric structures and sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to U.S. Pat. No. 5,623,065, herein incorporated by reference.

Example 5

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55° C. for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides may be analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis may be periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides may be purified by HPLC, as described by Chiang et al., J. Biol. Chem. 1991, 266, 18162-18171. Results obtained with HPLC-purified material may be similar to those obtained with non-HPLC purified material.

Example 6

Alternative Pathway Reconstitution

The alternative pathway was reconstituted with purified human proteins essentially as described by Keil (Keil et al., *Am J Hematol* 1995;50(4):254-62).

Example 7

Complement Activity Assay

Measurement of complement activity was accomplished by measuring C3 convertase activity by combining the following C3 (125 µg/ml), Factor B (20 µg/ml) and Factor D (0.2 µg/ml) in Hand's Balanced Salt Solution (HBSS)

30

µg/ml) Factor D (0.2 µg/ml), Factor H (25 µg/ml) and Factor I (2 µg/ml) in Hand's Balanced salt Solution (HBSS) buffered with 5 mM HEPES, pH 7.2. Incubations were carried out under ambient conditions in the presence of oligonucleotide concentrations (up to 300 µg/ml). Aliquots were removed at selected intervals and immediately diluted 50-fold in ice cold ELISA dilution buffer. Complement split products were measured by ELISA. Complement activation in serum was measured in both rhesus monkey and human serum as follows:

Dilutions of oligonucleotides were added to normal human or rhesus serum at a 1:10-1:20 ratio, v/v. The samples were incubated at 37° C. and aliquots removed at selected intervals. Complement activation was terminated by either placing the aliquots in an acid precipitating reagent for RIA determinations, or by diluting the aliquots 1:50 in ice cold sample diluent for ELISA determinations.

As controls for some experiments zymosan A (500 µg/ml) or cobra venom factor (CVF; 2 U/ml) were used to activate the alternative pathway in the presence of the oligonucleotide. Each was added at a final volume of 1:20.

Example 8

In vivo Activation of Complement

Cynomolgus monkeys received single doses of 2 to 20 mg/kg of oligonucleotide or a vehicle control solution by i.v. infusion for periods ranging from 2 to 120 minutes. Measurement:

The level of complement split products Bb, C3a, C4a and C5a was determined in EDTA plasma samples using commercially available (Amersham Life Sciences, Amersham, Little Chalfont, Buckinghamshire, England; Quidel, San Diego, Calif.) radioimmunoassay or enzyme-linked immunosorbent assay kits.

Total hemolytic complement activity in serum (CH50) was assayed in serum samples using the standard hemolytic assay (Harbeck et al., *Diagnostic Immunology Laboratory Manual*, pp 9-20, Raven Press, New York, 1991). Factor H concentrations in monkey plasma was determined by radial immunodiffusion (Harbeck et al.) using an anti-human Factor H antibody.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 1

<210> SEQ ID NO 1

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> GENE INFORMATION: Description of Artificial Sequence: Synthetic

<400> SEQUENCE: 1

gcccaagctg gcacccgtca

20

buffered with 5 mM HEPES, pH 7.2. Incubations may be carried out under ambient conditions in the presence of oligonucleotide (concentrations up to 300 µg/ml). Aliquots may be removed at selected intervals and immediately diluted 50-fold in ice cold ELISA dilution buffer. Complement split products may be measured by ELISA.

In another example measurement of complement activity was accomplished by measuring C3 convertase activity by combining the following C3 (125 µg/ml), Factor B (20

What is claimed is:

1. A method for modulating complement activation in a cell, tissue or a bodily fluid comprising independently administering to said cell, tissue or bodily fluid a first concentration and a second, independent concentration of an oligonucleotide which comprises one or more phosphorothioate modifications, wherein said oligonucleotide ini-

31

trates complement activation at said first concentration and inhibits complement activation at said second, independent concentration.

2. The method of claim 1, wherein said first concentration is less than or equal to 80 $\mu\text{g/ml}$.

32

3. The method of claim 1, wherein said second concentration is at least 200 $\mu\text{g/ml}$.

* * * * *

DOCUMENT-IDENTIFIER: US 5698449 A
TITLE: Synthetic peptide and its uses

BSPR:

The invention further provides a test kit for detecting or quantitating immune complexes comprising a container of the Clq fragment or a container of the synthetic peptide. Also, the synthetic peptide or Clq fragment may be used to treat an inflammatory response in a mammal by administering to the mammal at the site of inflammation an amount of the Clq fragment or synthetic peptide effective to inhibit the binding of immune complexes by Clq.

DEPR:

The Clq fragment or synthetic peptide can also be used as an anti-inflammatory drug. Inflammation in some diseases, such as rheumatoid arthritis, has been associated with the deposition of immune complexes in tissues and the activation of the complement cascade. It is the binding of Clq to immune complexes deposited in the tissues which initiates the complement cascade, and the action of the complement components, alone or concurrently with other biologic molecules, ultimately leads to tissue damage.

DEPR:

Accordingly, a Clq fragment or synthetic peptide according to the invention can be administered to a mammal suffering from inflammation mediated by the classical complement pathway to inhibit the binding of immune complexes by Clq and, thereby, prevent tissue damage and further inflammation. The Clq fragments or synthetic peptides are preferably injected at the site of inflammation in the mammal in order to obtain an adequate local concentration of Clq fragments or synthetic peptides. The advantage of such a therapeutic approach is that small peptides are less likely to illicit an immune response

which would render the drug inactive.

DEPR:

The resulting inhibition curve is presented in FIG. 6. The 50% inhibition concentration was approximately 10 μ M, which was an order of magnitude higher than the 50% inhibition concentration for CBP2 inhibiting Protein A. The 50% inhibition concentration for liquid phase Clq inhibiting solid phase Clq was approximately 10 nM or 1000 fold less than the CBP2 concentration (see FIG. 6).

DEPR:

4. Equal molar concentrations of luteinizing hormone releasing hormone (LHRH) (Beckman Instruments, Palo Alto, Calif.) or various concentrations of Protein A were substituted for CBP2 in the inhibition assay described in Part B. In the case of the Clq inhibition experiments, LHRH and Clq were substituted for CBP2 as a negative and a positive control, respectively. The use of LHRH also tested whether the inhibition of Protein A or Clq by CBP2 was due to CBP2 specifically, or simply due to nonspecific effects attributable to a small peptide. The results are shown in FIGS. 5 and 6. As indicated in these figures, LHRH demonstrated no Protein A or Clq inhibitory activity over the same concentration range examined for CBP2, and both Protein A and Clq were inhibitory, as expected.

DEPR:

Based on the inhibition data in conjunction with the above controls, it can be concluded that CBP2 inhibits Clq and Protein A binding of Ig-HRP through a binding interaction with solution phase Ig-HRP. The results indicate that CBP2 binds immunoglobulin in a specific manner.

DEPR:

A 1 ml sample of 20 μ g/ml PAP was loaded on an equilibrated CBP2 column (prepared as described in Example 3) and allowed to circulate

continuously for 15 min over the column. The column was then washed with 12 ml Clq buffer, followed by 3 ml of 336.2 $\mu\text{g/ml}$ CBP2 (this CBP2 concentration was 20 times the concentration of CBP2 needed to give 50% inhibition Ig-HRP binding to Clq) diluted in Clq buffer. An additional 6 ml of Clq buffer was used to wash the column followed by 3 ml of 2% (v/v) acetic acid and then with 6 ml of Clq buffer to completely wash the column. Fractions were collected and assayed for the presence of peroxidase activity as described above in Part A. A control column was run as described above, except that 3 ml of LHRH at an equimolar concentration as the CBP2 solution, was substituted for the CBP2 wash.

DEPR:

To address the question of nonequilibrium conditions, a 1 ml sample of 20 $\mu\text{g/ml}$ PAP plus 200 $\mu\text{g/ml}$ CBP2 (0.1 mM; a ten times greater concentration than that required to give 50% inhibition of Ig-HRP binding to Clq) or 152 $\mu\text{g/ml}$ LHRH (0.1mM) was incubated for 1 hour at 25.degree. C. and then loaded on a pre-equilibrated CBP2 column and allowed to pass continuously over the column for 15 min. The sample was then eluted with 21 ml Clq buffer followed by 3 ml of 2% (v/v) acetic acid and then 6 ml Clq buffer. The column fractions were assayed for the presence of peroxidase as described above in Part A, and the concentration of PAP was calculated from the standard curve data.

DEPL:

D. CBP2 Inhibition of Solid Phase Clq.

DEPL:

E. Control Experiments For CBP2 Inhibition Of Protein A And Clq



US005698449A

United States Patent [19]**Baumann et al.**[11] **Patent Number:** **5,698,449**[45] **Date of Patent:** **Dec. 16, 1997**[54] **SYNTHETIC PEPTIDE AND ITS USES**[75] **Inventors:** Michael A. Baumann, Baltimore, Md.;
Byron E. Anderson, Morton Grove, Ill.[73] **Assignee:** Northwestern University, Evanston,
Ill.[21] **Appl. No.:** 335,049[22] **Filed:** Nov. 7, 1994**Related U.S. Application Data**[62] **Division of Ser. No. 598,416, Oct. 16, 1990, Pat. No.**
5,364,930.[51] **Int. Cl.⁶** **G01N 33/564**[52] **U.S. Cl.** **436/507; 436/518; 436/538;**
436/807; 514/14; 530/326; 530/327; 530/413[58] **Field of Search** **436/507, 535,**
436/538, 541, 539, 518, 807; 514/12, 13,
14, 15; 530/326, 327, 328, 413, 415[56] **References Cited****U.S. PATENT DOCUMENTS**

3,941,763	3/1976	Sarantakis	260/112.5
4,546,082	10/1985	Knijan et al.	435/172.3
4,588,684	5/1986	Brake	435/68
4,775,622	10/1988	Hitzeman et al.	435/68
4,945,039	7/1990	Suzuki et al.	435/7
5,084,398	1/1992	Huston et al.	436/535
5,364,930	11/1994	Baumann et al.	530/326

OTHER PUBLICATIONS

Blair, A.H., and Ghose, T.I., *J. Immunol. Methods*, 59:129-143 (1983).
 Burton, *Molec. Immunol.*, 22, 161-206 (1985).
 Burton, *Molec. Immunol.*, 25, 1175-81 (1988).
 Burton et al., *Nature*, 288, 338-44 (1980).
 Chou and Fasman, *Adv. Enzym.*, 47, 45-148 (1978).
 Davis et al., *Biochem. Int'l.*, 10, 395-404 (1985).
 DeGrado et al., *J. Am. Chem. Soc.*, 103, 679-81 (1981).
 Duncan and Winter, *Nature*, 332, 738-40 (1988).
 Ellman, *Arch. Biochem. Biophys.*, 74, 443-450 (1958).
 Ellman, *Arch. Biochem. Biophys.*, 82, 70-77 (1959).
 Fields, *Meth. Enzymol.*, 25B, 464-68 (1972).
 Garnier et al., *J. Molec. Biol.*, 120, 97-120 (1978).
 Gauthier et al., *J. Exp. Med.*, 156:766-777 (1982).
 Geusdon et al., *J. Histochem. Cytochem.*, 27, 1131-39 (1979).
 Hughes-Jones and Gardner, *Immunology*, 34, 459-63 (1978).
 Jones et al., *J. Immunol. Meth.*, 53, 201-208 (1982).
 Kulkarni, P.N., Blair, A.H., and Ghose, T.I., *Cancer Res.*, 41:2700-06 (1981).
 Lancet et al., *Biochem. Biophys. Res. Commun.*, 85, 608-614 (1978).
 Langone et al., *Adv. Immunol.*, 32, 157-252 (1982).

Langone et al., *J. Immunol.*, 121, 327-32 (1978).
 Lukas et al., *J. Immunol.*, 127, 2555-60 (1981).
 Marquart et al., *J. Mol. Biol.*, 141, 369-91 (1980).
 Merrifield, *J. Am. Chem. Soc.*, 85, 2149-54 (1963).
 Moe and Kaiser, *Biochem.*, 24, 1971-76 (1985).
 Prystowsky et al., *Biochemistry*, 20, 6349-56 (1981).
 Reid, *Biochemical J.*, 179, 367-71 (1979).
 Reid et al., *Biochemical J.*, 203, 559-69 (1982).
 Stalinhem et al., *Immunochem.*, 10, 501-507 (1973).
 Taylor et al., *Mol. Pharmacol.*, 22, 657-66 (1982).
 Theofilopoulos and Dixon, *Adv. Immunol.*, 28, 89-220 (1979).
 Baumann and Anderson, *J. Biol. Chem.*, 265, 18414 (Oct. 25, 1990).
 Veerhuis et al., *Eur. J. Immunol.*, 15, 881-887 (1985).
 Endo et al., "An Enzyme-Linked Immunoassay for the Measurement of Circulating Immune Complexes." *Ann. Clin. Biochem.* (1983) 20:163-168.
 Marqués et al., "Arginine Residues of the Globular Regions of Human Clq Involved in the Interaction with Immunoglobulin G," *J. Biol. Chem.* (1993) 268:10393-02.
 Sellar et al., "Characterization and Organization of the Genes Encoding the A-, B- and C-chains of Human Sub-component Clq," *Biochem. J.* (1991) 74:481-490.
 Comis et al., "Evidence for Arginine Residues in the Immunoglobulin-binding Sites of Humans Clq", *Biochim. Biophys. Acta*, 842(1):45-51, 1985.

Primary Examiner—Carol A. Spiegel
Attorney, Agent, or Firm—Sheridan Ross

[57] **ABSTRACT**

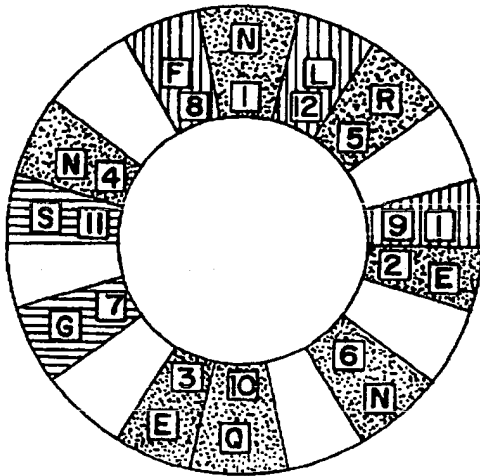
The invention provides a fragment of C1q which is characterized in that a plurality of such fragments selectively binds immune complexes or aggregated immunoglobulins in the presence of monomeric immunoglobulin. The invention also provides a synthetic peptide comprising the sequence:

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln Ala Thr (SEQ ID NO 2)
 1 5 10

or variants thereof capable of binding immunoglobulin. Like the C1q fragment, a plurality of the peptides can selectively bind immune complexes or aggregated immunoglobulins in the presence of monomeric immunoglobulin. As a result of this property, the fragments and peptides are well-adapted for removing immune complexes and aggregated immunoglobulins from fluids containing monomeric immunoglobulin, and for detecting or quantitating immune complexes in such fluids. The invention also provides a binding material for removing immune complexes or aggregated immunoglobulins from a fluid. The binding material comprises plural binding peptides, the peptides being characterized in that a plurality of them selectively binds immune complexes and aggregated immunoglobulins in the presence of monomeric immunoglobulin.

6 Claims, 15 Drawing Sheets

FIG. 2

B. Protein A Fragment B
(Residues 142-153)

CBP 2

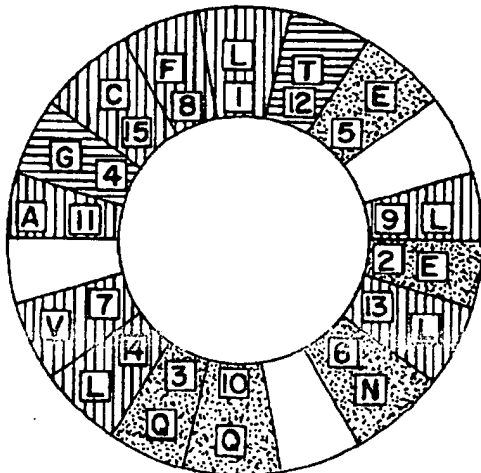
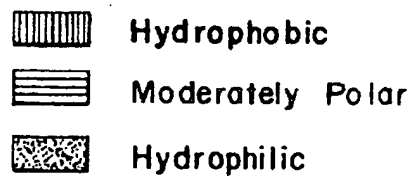
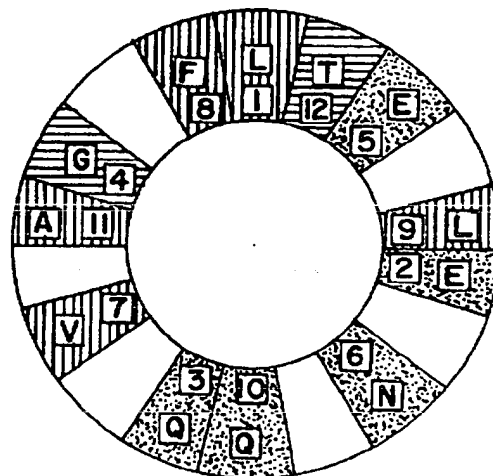
Clq B Chain Helix:
(Residues 189-200)

FIG. 3

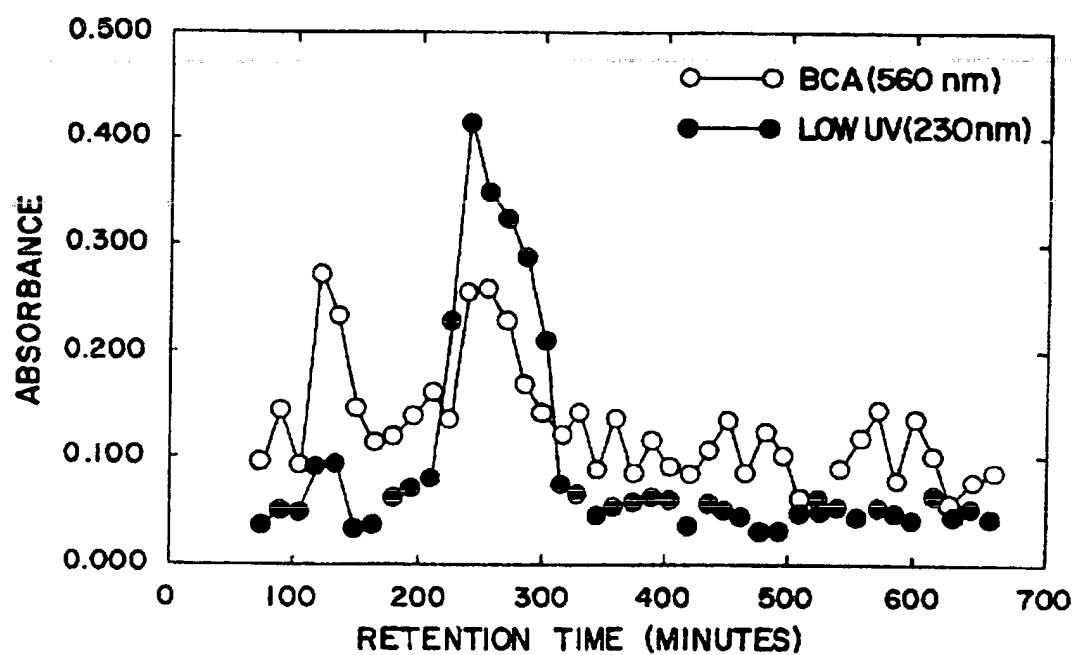


FIG. 4A

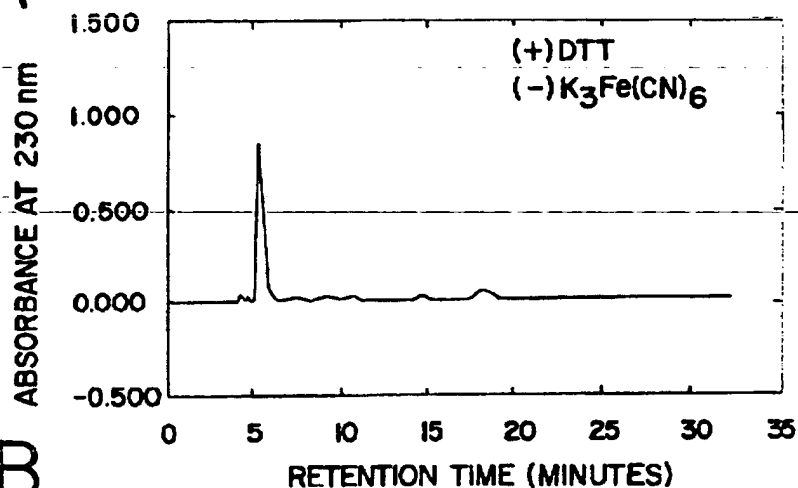


FIG. 4B

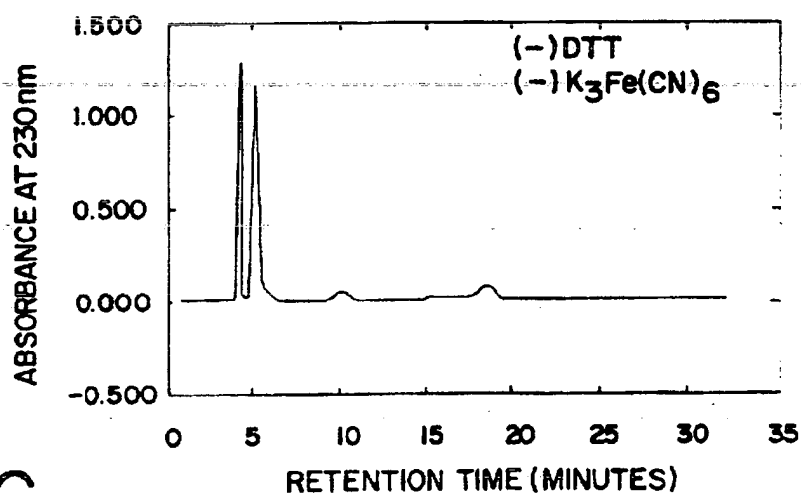


FIG. 4C

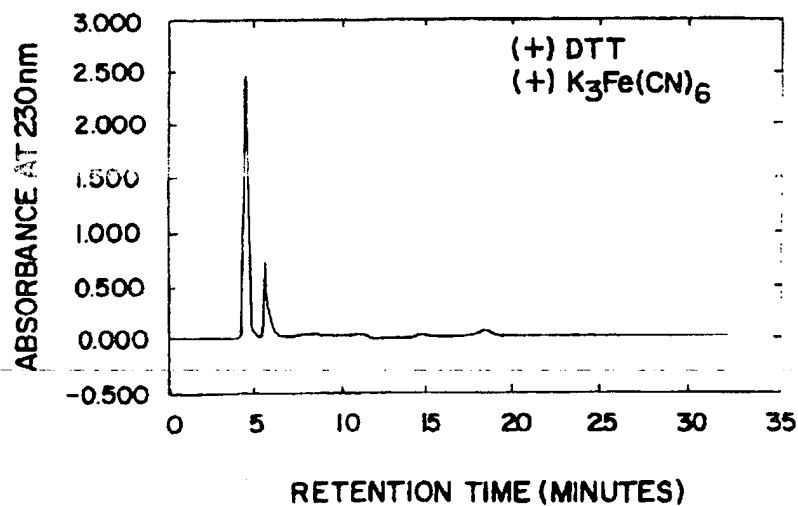


FIG. 5

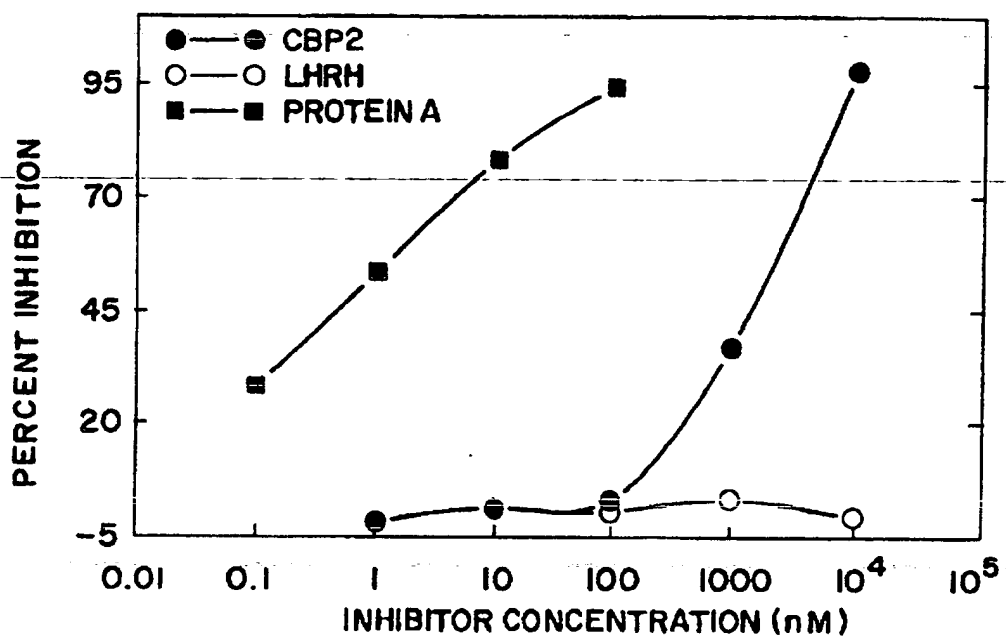


FIG. 6

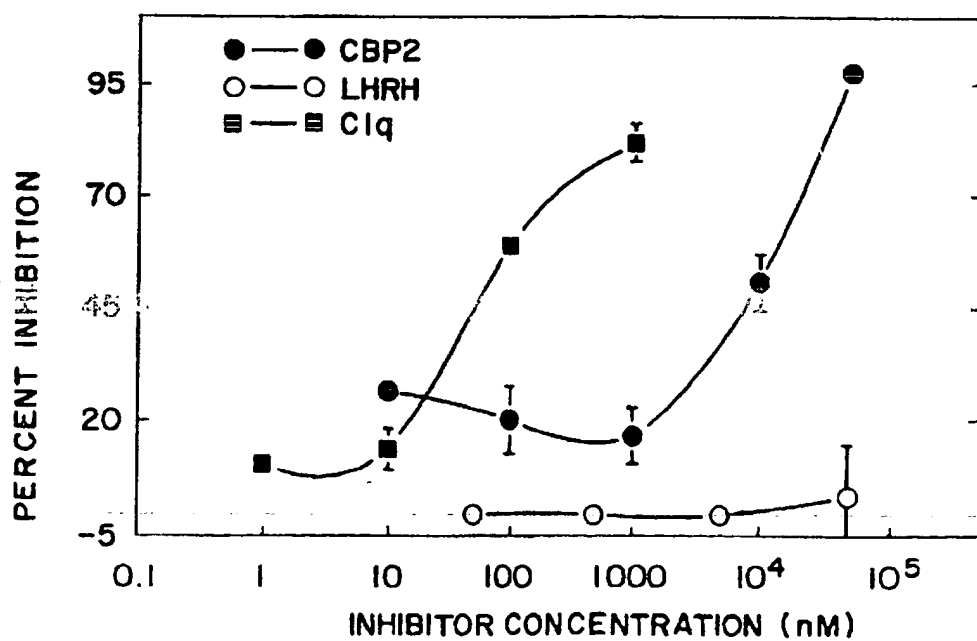


FIG. 7

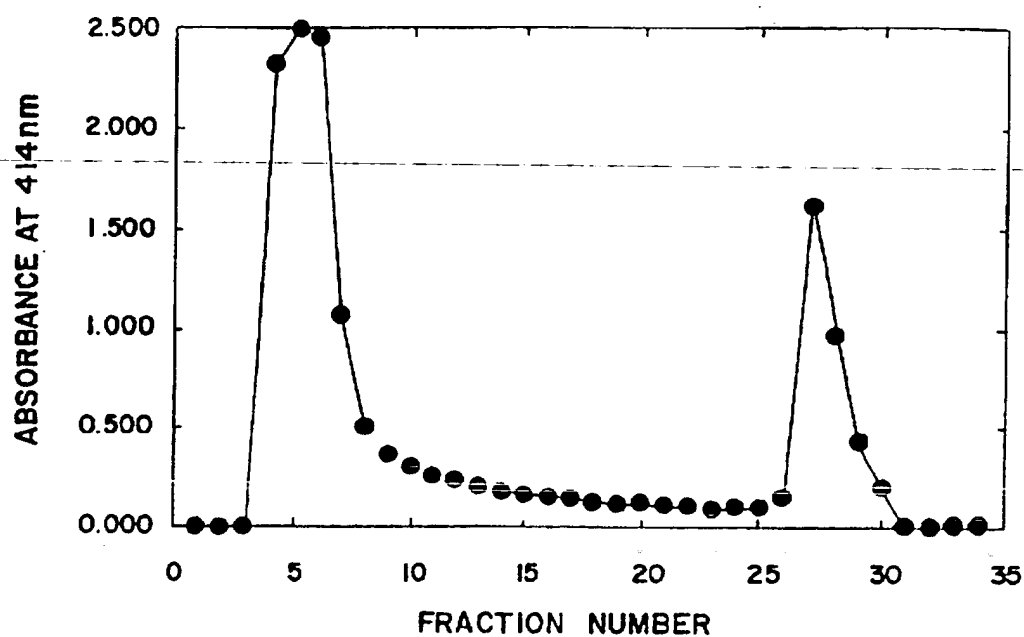


FIG. 8

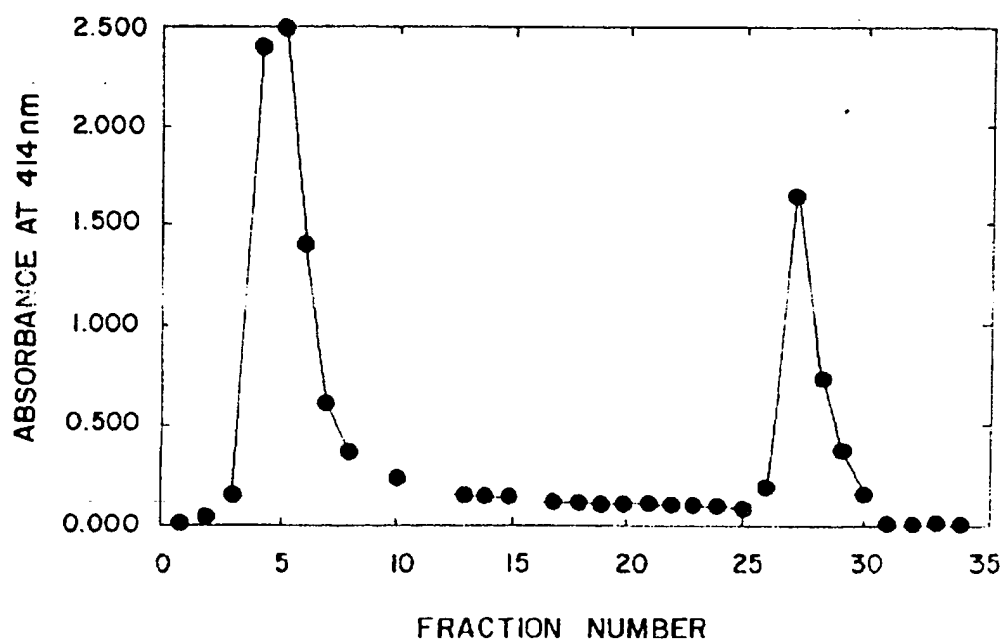


FIG. 9A

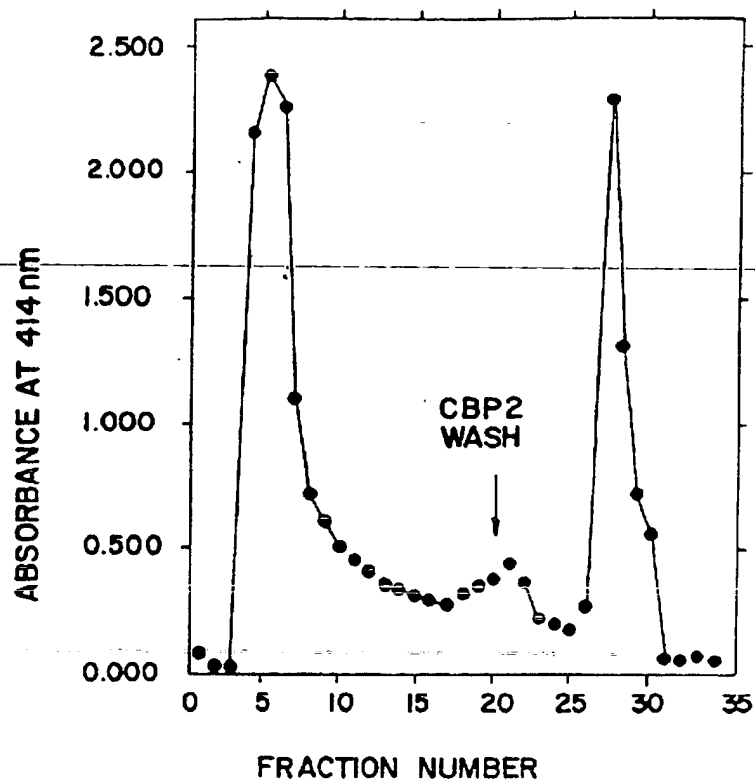


FIG. 9B

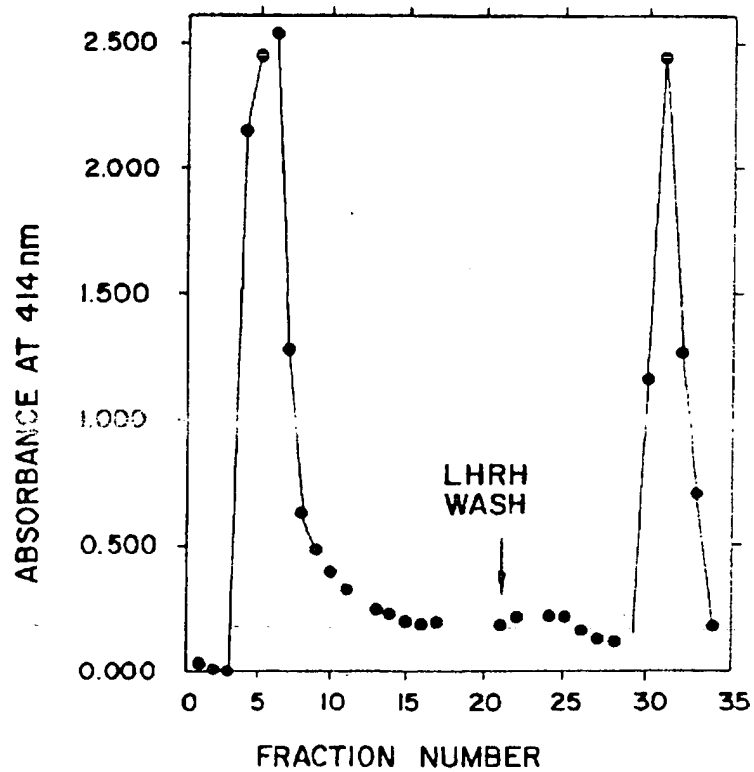


FIG. 10

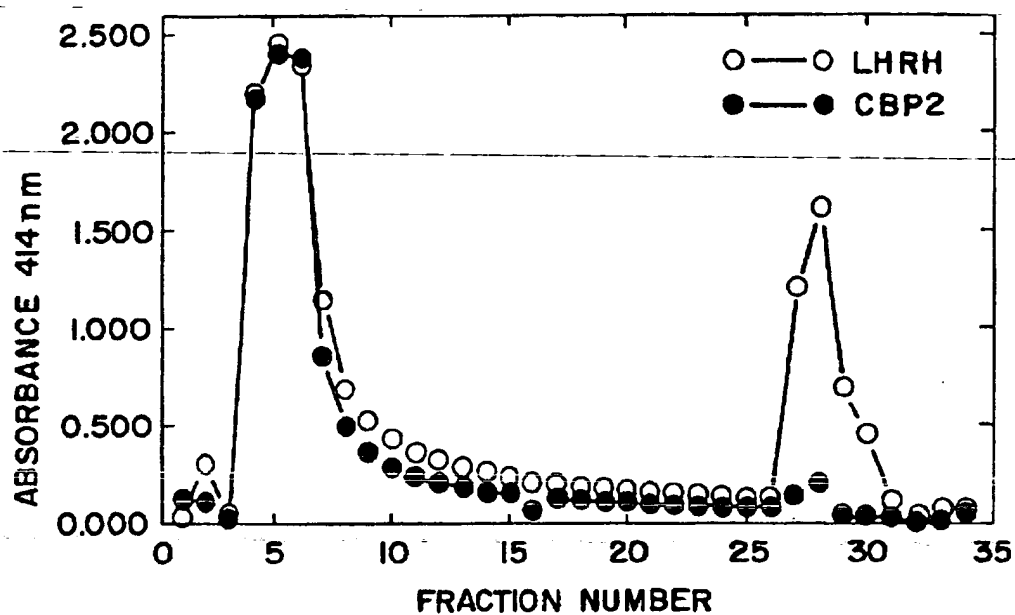


FIG. II

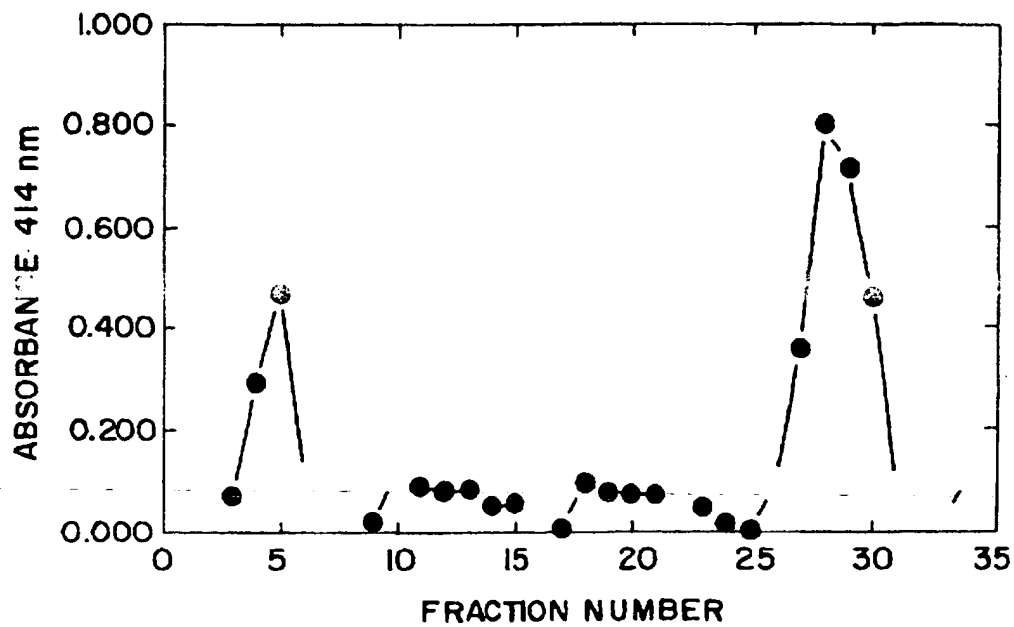


FIG. 12

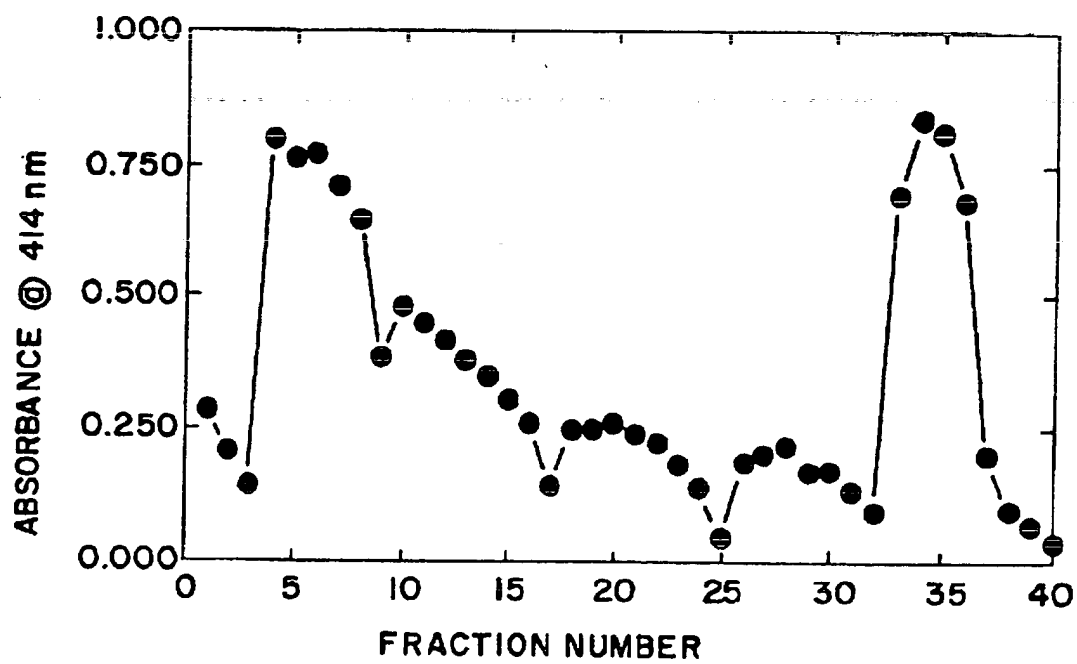


FIG. 13A

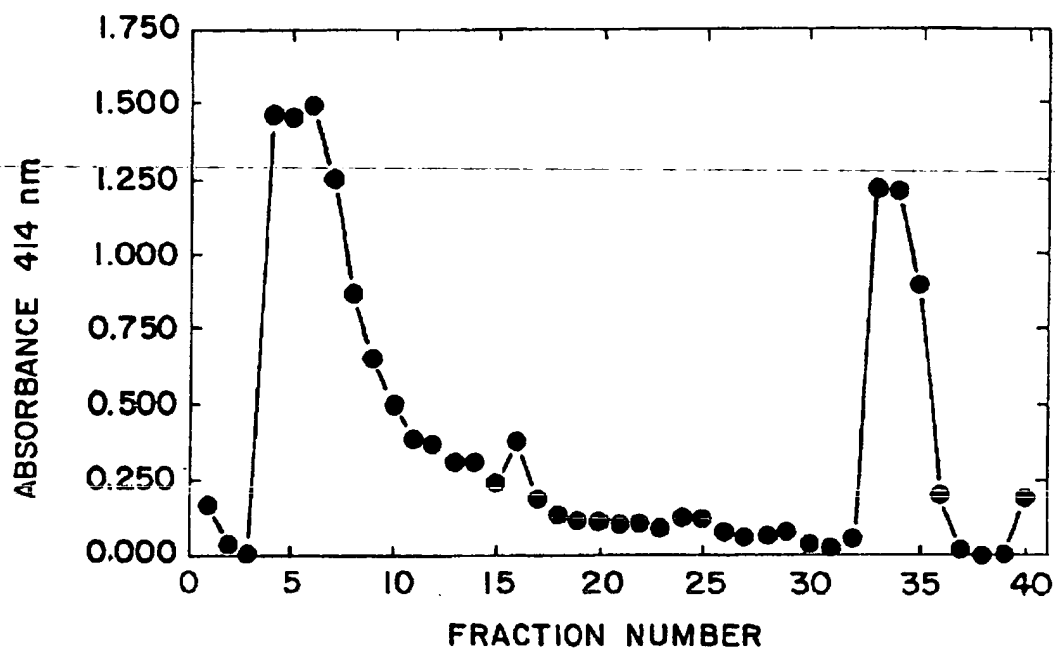


FIG. 13B

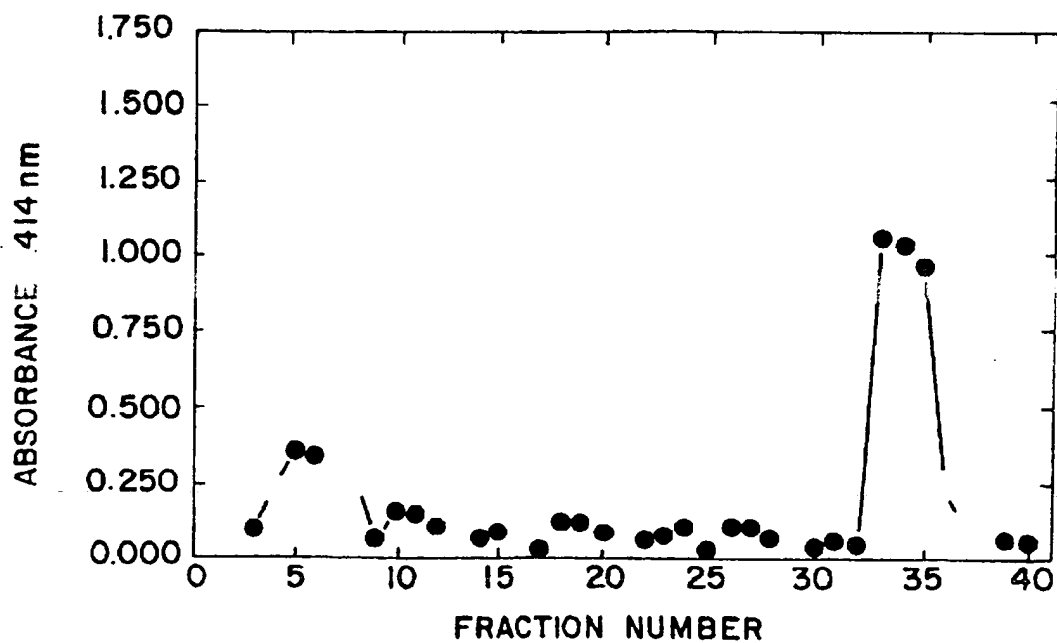


FIG. 13C

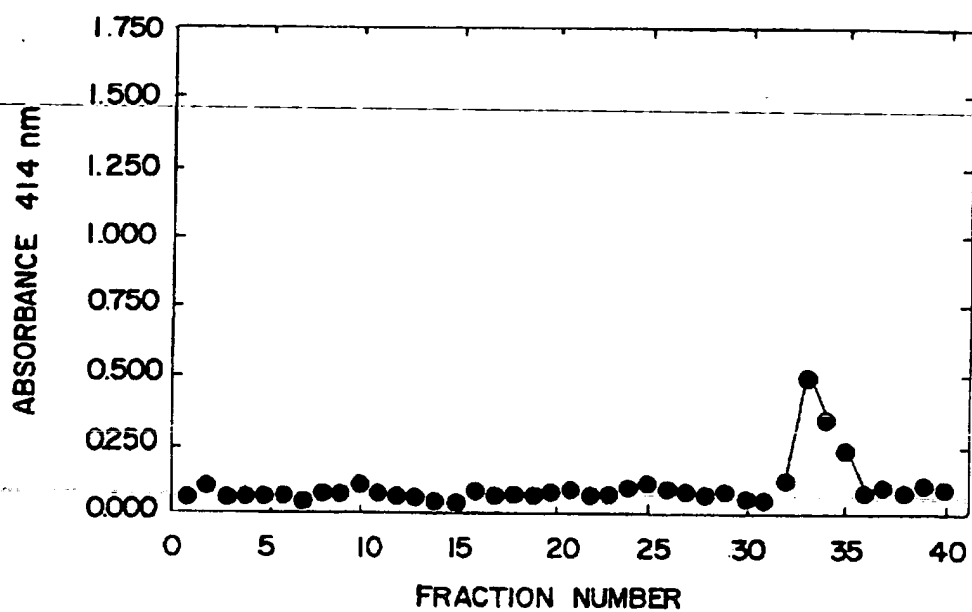


FIG. 14A

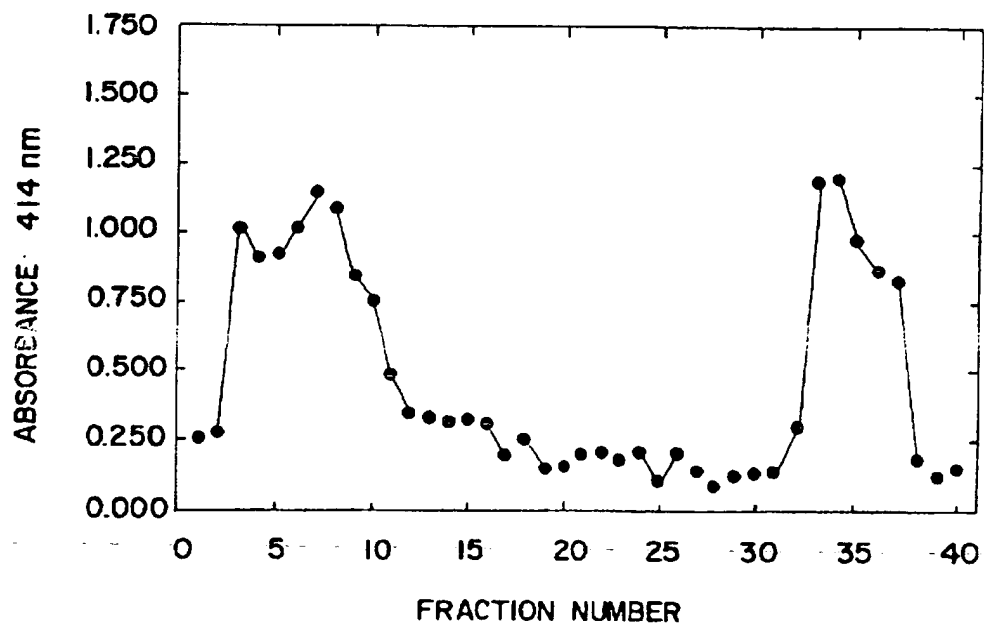


FIG. 14B

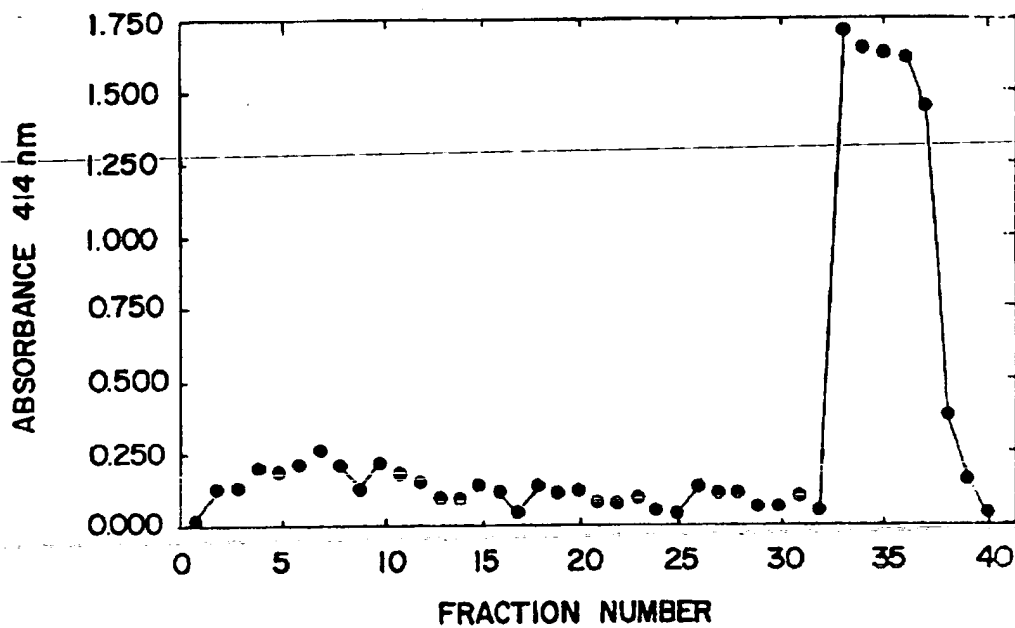


FIG. 14C

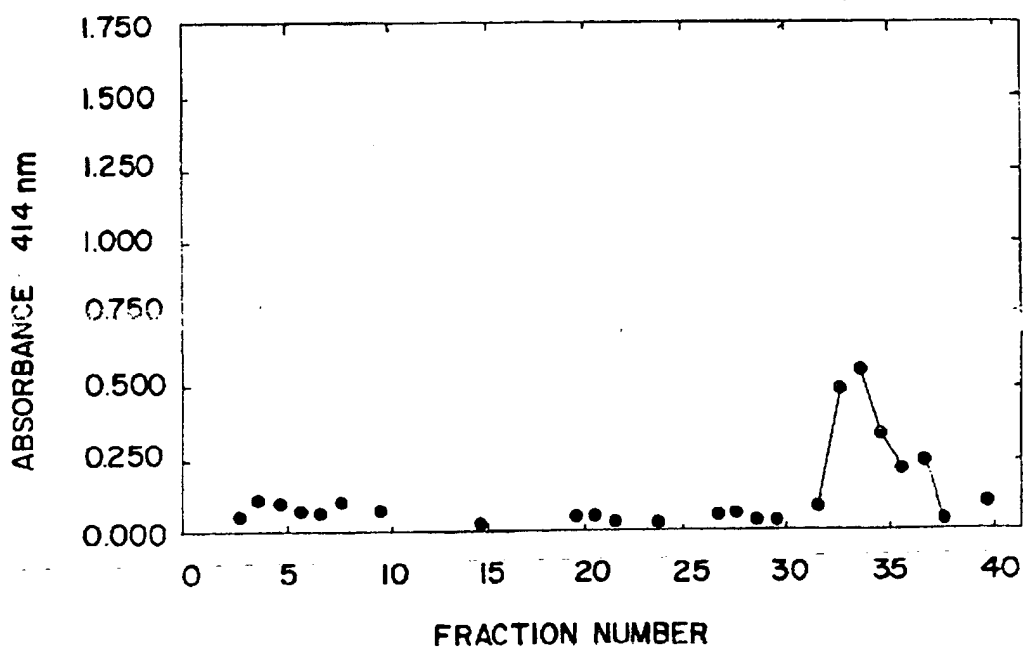


FIG. 15

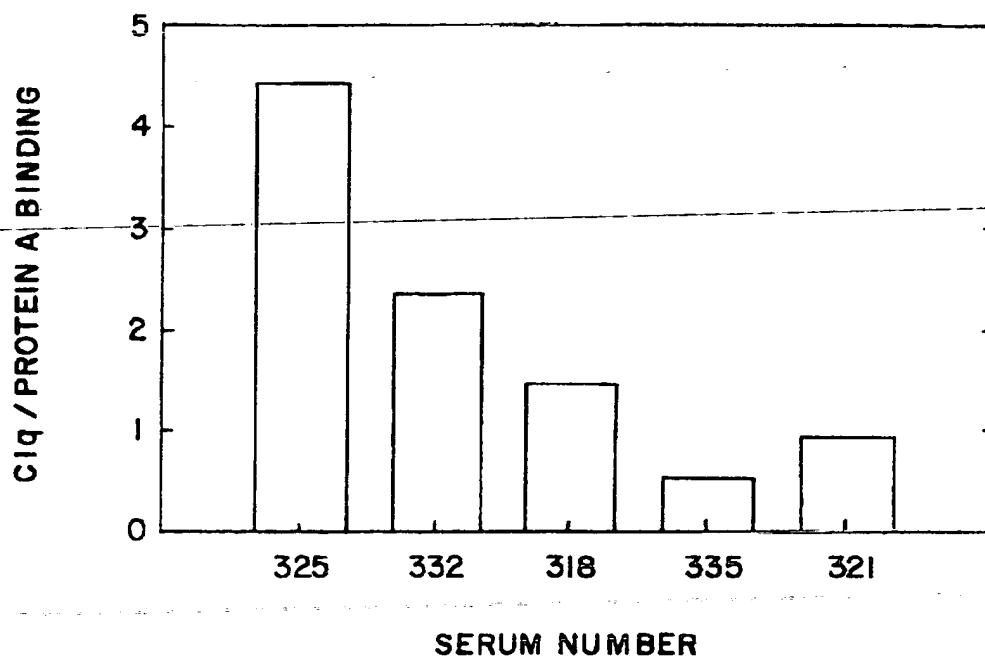


FIG. 16

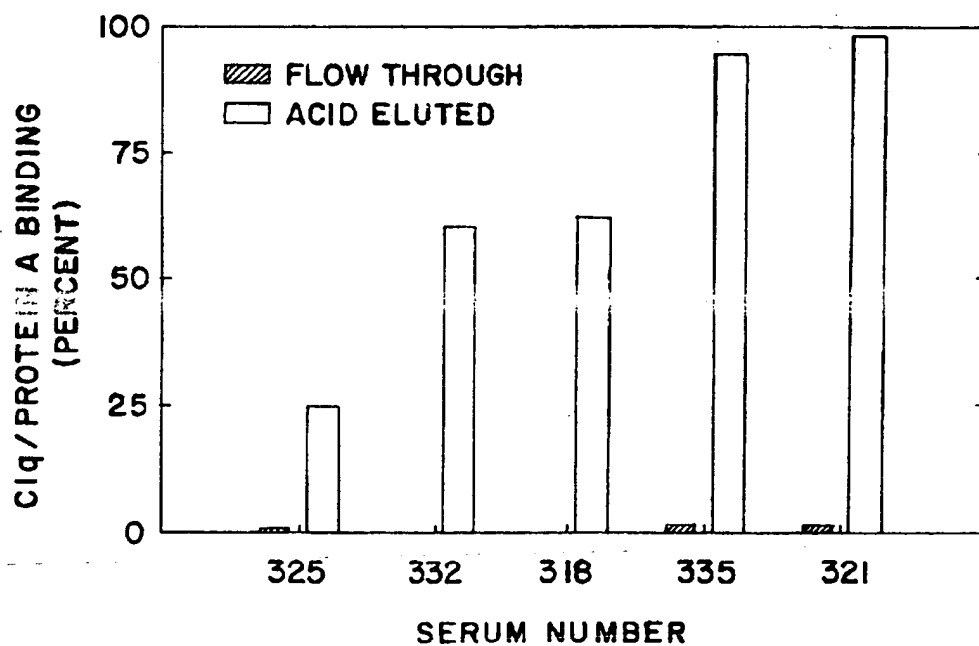


FIG. 17

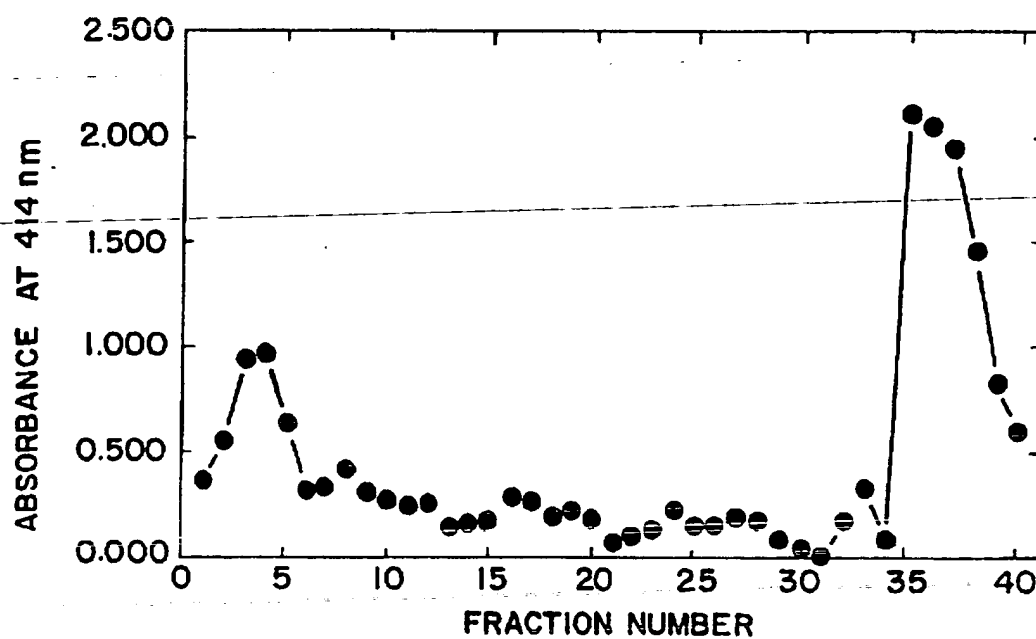


FIG. 18

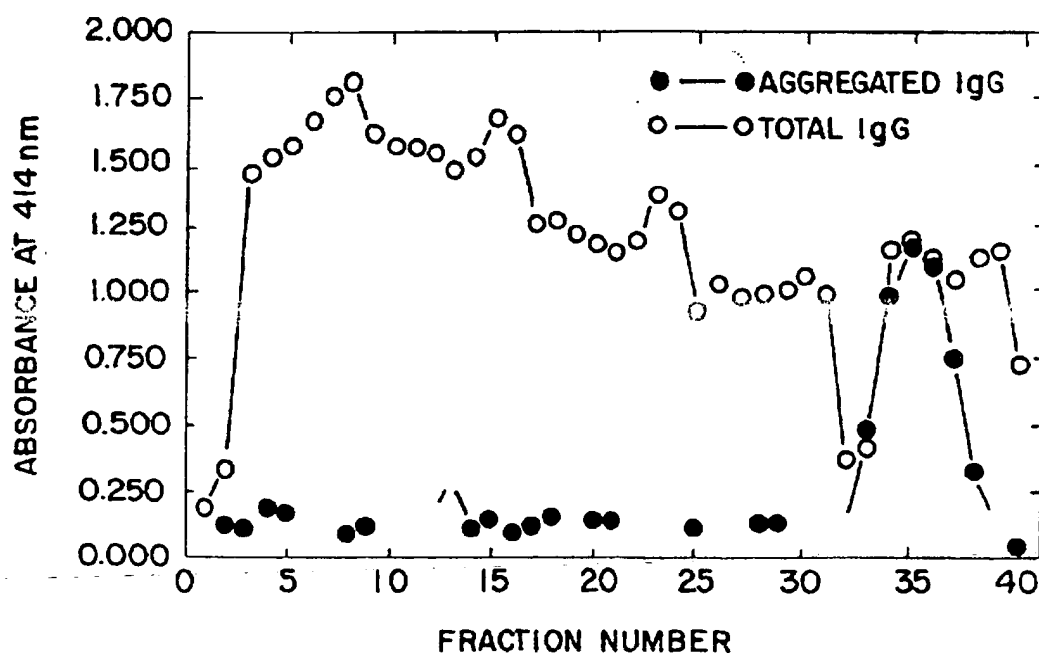
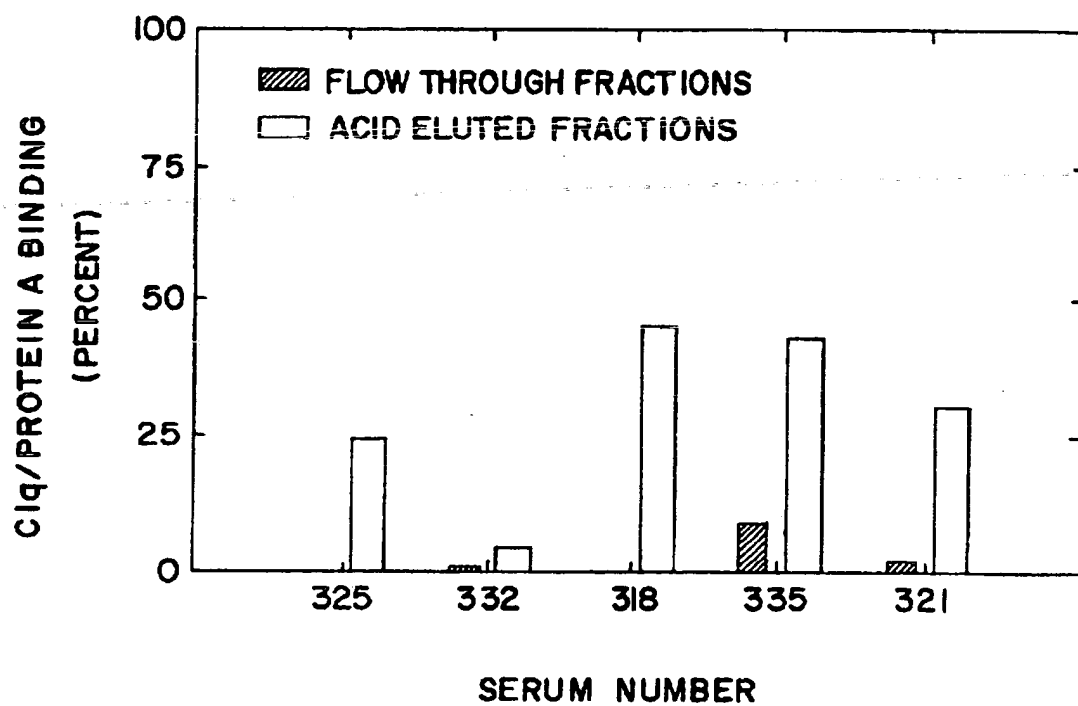


FIG. 19



SYNTHETIC PEPTIDE AND ITS USES

This application is a division of application Ser. No. 07/598,416, filed Oct. 16, 1990, now U.S. Pat. No. 5,364,930.

FIELD OF THE INVENTION

This invention relates to a fragment of C1q and to a synthetic peptide and to their use to selectively bind immune complexes and aggregated immunoglobulins. The invention also relates to a binding material which can be used to selectively remove immune complexes and aggregated immunoglobulins from fluids.

BACKGROUND OF THE INVENTION

An immune complex is an aggregate of immunoglobulins, non-immunoglobulin serum proteins, and antigens. Immune complexes are formed as a natural consequence of the immune response to antigens of infectious agents, to normal tissue components in the case of autoimmune diseases, to tumor-associated antigens, and to other antigens. The complexes are normally removed by the cells of the reticuloendothelial system. When this system is compromised or overloaded, circulating immune complexes may deposit in a number of organs, thereby causing possibly severe clinical problems. Further, in cancer, it is postulated that immune complexes may block other effector mechanisms of the immune system which would otherwise destroy malignant cells. Several studies have indicated that removal of circulating immune complexes may be an effective therapeutic technique. See, e.g., Theofilopoulos and Dixon, *Adv. Immunol.*, 28, 90-220 (1979); Theofilopoulos and Dixon, *Immunodiagnosis of Cancer*, page 896 (M. Decker Inc., New York, N.Y. 1979).

Immune complexes form as a result of immunoglobulins reacting with antigens. Immunoglobulins are able to cross-link antigens so that a lattice network of immunoglobulins bound to antigens is formed. Once the antigen-immunoglobulin reaction has occurred, the immune complex can then be decorated with a variety of serum proteins such as the proteins of the complement cascade.

Complement component C1q selectively binds immune complexes in the presence of monomeric immunoglobulin because of the molecule's ability to develop a "functional affinity" when binding immune complexes. A "functional affinity" results when multiple low affinity receptors, confined in space, interact with multiple ligands, which are also confined in space. Normally, an individual ligand would rapidly associate and dissociate from the low affinity receptor but, when multiple ligands in a complex interact with multiple receptors, the dissociation from the receptors is very slow since the probability of all ligands dissociating at the same time is very low. The slower dissociation rate results in an affinity several orders of magnitude greater than the individual receptor's affinity. The difference in affinities for the individual ligand and the complexed ligand produces a selection for the complexed ligand when presented with both species.

The mature C1q molecule contains two distinct portions, the stalk and the globular head. There are six globular head regions per C1q molecule. Each contains a low affinity immunoglobulin binding site. Hughes-Jones and Gardner, *Immunology*, 34, 459-63 (1978); Duncan and Winter, *Nature*, 332, 738-40 (1988). Since there are six globular head regions on C1q, the molecule can form multiple binding interactions with the multiple immunoglobulins

present in immune complexes. Id. The result is a higher net affinity for immune complexes (id.) due to the low probability of more than one bound globular head receptor dissociating simultaneously (i.e., a functional affinity develops). Thus, when C1q is presented with both immune complexes and monomeric immunoglobulin, it selectively binds to the immune complexes because of the slower dissociation kinetics of the immune complexes.

Many investigators have tried to identify the residues on immunoglobulins that are recognized by C1q. Initial theoretical studies that compared the sequences of immunoglobulin Fc regions of various species known to bind human C1q produced four possible sites in two general locations: 1) the residues flanking Trp277 and Tyr278 (residues 275-295) (Lukas et al., *J. Immunol.*, 127, 2555-60 (1981); Prystowsky et al., *Biochemistry*, 20, 6349-56 (1981)); and 2) the residues flanking Glu318 (residues 316-338) (Stalinheim et al., *Immunochem.*, 10, 501-507 (1973); Burton et al., *Nature*, 288, 338-44 (1980)). Various studies by authors advocating one or the other site produced conflicting results.

However, Duncan and Winter recently performed a series of more conclusive experiments. Duncan and Winter, *Nature*, 332, 738-40 (1988). Using recombinant DNA techniques, they were able to systematically alter the various residues of the two disputed sites. Then, by determining the ability to bind C1q of each of the resulting immunoglobulins, the actual site and specific binding residues were determined. They localized the core of the C1q interactions to residues 318, 320, and 322 in the Fc region of human IgG. Despite the success of Duncan and Winter, the site on immunoglobulins where C1q binds may not be limited to the residues indicated by their work. In fact, other immunoglobulin residues may also be involved in the C1q-immunoglobulin interaction that could not be detected using their approach. This will not be resolved until high resolution x-ray diffraction data are obtained for the C1q-Fc region complex and the complete binding interaction is determined.

Bacterial proteins such as *Staphylococcus aureus* Protein A also bind to the immunoglobulin Fc region. Unlike C1q, the Protein A-immunoglobulin interaction is understood in detail. In a series of crystallographic studies by Deisenhofer, et al., the structure of human IgG Fc, Protein A, and finally the IgG Fc-Protein A Fragment B co-crystal were determined. Deisenhofer et al., *Hoppe-Seyler's Z. Physiol. Chem. Bd.*, 359, S. 975-85 (1978); Marquart et al., *J. Mol. Biol.*, 141, 369-91 (1980). One of the most important pieces of information to come from this structure is the exact contact residues involved in the interaction. Those residues are Met 252, Ile 253, Ser 254, Val 308, Leu 309, His 310, Gln 311, Asn 312, His 433, Asn 434, His 435, and Tyr 436 of the human IgG Fc region. These residues are located at the interface between the CH2 and CH3 regions of the Fc portion of IgG, and some of them (309-312) are in close proximity to the proposed immunoglobulin binding site for C1q (318, 320 and 322).

Unlike C1q, Protein A binds to the Fc portion of immunoglobulins with high affinity. Ellman, *Arch. Biochem. Biophys.*, 74, 443-450 (1958). Thus, Protein A cannot differentiate between complexed and monomeric immunoglobulins.

However, PCT application WO 89/04675 teaches the preparation of analogs of Protein A that have a lower affinity for the Fc region and which can develop a functional affinity for immune complexes when arrayed in a specific manner. The analogs are analogs of a binding domain of Protein A or of related sequences from functionally similar bacterial

proteins such as Protein G (see page 10). This PCT application reports that oligomers of the analogs, or an array of the analogs disposed about the surface of an insoluble matrix, develop a functional affinity for immune complexes.

SUMMARY OF THE INVENTION

The invention provides a fragment of C1q which is characterized in that a plurality of such fragments selectively binds immune complexes or aggregated immunoglobulins in the presence of monomeric immunoglobulin. The fragment can be used to bind immune complexes or aggregated immunoglobulins. It can also be used to detect or quantitate immune complexes or to remove immune complexes or aggregated immunoglobulins from fluids. In particular, since a plurality of the fragments can selectively bind immune complexes or aggregated immunoglobulins in the presence of monomeric immunoglobulin, the fragment is especially well-adapted for removing immune complexes and aggregated immunoglobulin from fluids containing monomeric immunoglobulin and for detecting or quantitating immune complexes in such fluids.

The invention also provides a synthetic peptide comprising the sequence:

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln Ala Thr [SEQ ID NO 2]
1 5 10

or variants thereof capable of binding immunoglobulin. The synthetic peptide can be used to bind immune complexes or aggregated immunoglobulins, to detect or quantitate immune complexes, or to remove immune complexes or aggregated immunoglobulins from fluids. Like the C1q fragment, a plurality of the peptides can selectively bind immune complexes or aggregated immunoglobulins in the presence of monomeric immunoglobulin. As a result of this property, the peptides of the invention are also well-adapted for removing immune complexes and aggregated immunoglobulin from fluids containing monomeric immunoglobulin, and for detecting or quantitating immune complexes in such fluids.

The C1q fragment and the synthetic peptide can be prepared in a number of ways, including using recombinant DNA techniques. Accordingly, the invention also comprises a DNA molecule encoding the C1q fragment or the synthetic peptide, a vector comprising the DNA molecule operatively linked to expression control sequences, and a host cell transformed with the vector. The C1q fragment or synthetic peptide can be prepared by culturing the transformed host cell.

The invention further provides a test kit for detecting or quantitating immune complexes comprising a container of the C1q fragment or a container of the synthetic peptide. Also, the synthetic peptide or C1q fragment may be used to treat an inflammatory response in a mammal by administering to the mammal at the site of inflammation an amount of the C1q fragment or synthetic peptide effective to inhibit the binding of immune complexes by C1q.

Finally, the invention provides a binding material for removing immune complexes or aggregated immunoglobulins from a fluid. The material comprises plural binding peptides, the peptides being characterized in that a plurality of them selectively binds immune complexes and aggregated immunoglobulins in the presence of monomeric immunoglobulin. Preferably, the binding peptides are the C1q fragments of the invention or synthetic peptides comprising the sequence

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln Ala Thr [SEQ ID NO 2]
1 5 10

or a combination of the two. Immune complexes and aggregated immunoglobulins can be removed from fluids containing them by contacting the fluids with the binding material at a temperature and for a time sufficient to bind the immune complexes and aggregated immunoglobulins to the material, and then separating the fluid from the material. The invention also provides a device for removing immune complexes or aggregated immunoglobulins from a fluid comprising the binding material and a means for encasing the material so that the fluid can be contacted with it.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Shown are the sequences (from top to bottom) of *S. aureus* Protein A fragment B, the predicted helical region of C1q, and CBP2 (Peptide (SEQ ID NO 3)).

FIG. 2: Helical wheel diagrams for the helical region of Protein A (residues 142-153), predicted helical region of C1q B chain (residues 189-200), and CBP2.

FIG. 3: Shows the elution profile of CBP2 on a Sephadex G25 column.

FIG. 4A: Reverse phase High Performance Liquid Chromatography (HPLC) chromatograph of CBP2 under reducing conditions.

FIG. 4B: Reverse phase HPLC chromatograph of CBP2 under nonreducing conditions.

FIG. 4C: Reverse phase HPLC chromatograph of CBP2 in the presence of an oxidizing agent to favor the formation of disulfide linked CBP2 peptides.

FIG. 5: Shows the inhibition of the binding of immunoglobulin labeled with horseradish peroxidase (Ig-HRP) to solid phase Protein A by CBP2, Protein A, or luteinizing hormone releasing hormone (LHRH).

FIG. 6: Shows the inhibition of the binding of rabbit Ig-HRP to solid phase C1q by CBP2, C1q, or LHRH.

FIG. 7: The elution profile of horseradish peroxidase/anti-horseradish peroxidase immune complexes (PAP) passed over the HiPAC™ LTQ-CBP2 column.

FIG. 8: The elution profile of a mixture of PAP plus monomeric rabbit IgG.

FIG. 9A: Elution of PAP on a CBP2 column washed with solution of CBP2.

FIG. 9B: Elution profile of PAP on a CBP2 column washed with a solution of LHRH.

FIG. 10: The elution profile of PAP on the HiPAC™ LTQ-CBP2 column when PAP were incubated with either CBP2 or LHRH prior to being loaded on the column.

FIG. 11: The elution profile of aggregated human IgG on the HiPAC™ LTQ-CBP2 column.

FIG. 12: The elution profile of aggregated human IgG plus monomeric human IgG.

FIG. 13A: The elution profile of biotinylated aggregated human IgG plus monomeric human IgG (total IgG).

FIG. 13B: The elution profile of biotinylated aggregated human IgG.

FIG. 13C: The elution profile of C1q binding material.

FIG. 14A: The elution profile of biotinylated aggregated human IgG in diluted pooled normal human plasma (total IgG).

FIG. 14B: Elution profile of aggregated IgG.

FIG. 14C: Elution profile of C1q binding material.

FIG. 15: Graph of the ratio of C1q binding material to Protein A binding material for five patient sera prior to passage over the HiPAC™ LTQ-CBP2 column.

FIG. 16: Graph of the ratio of C1q binding material to Protein A binding material for five patient sera after passage over the HiPAC™ LTQ-CBP2 column.

FIG. 17: Shows the elution profile of aggregated human IgG diluted in C1q buffer and passed over the HiPAC™ FPLC-CBP2 column.

FIG. 18: Shows the elution profile of biotinylated aggregated human IgG diluted in normal human plasma and passed over the HiPAC™ FPLC-CBP2 column.

FIG. 19: Graph of the ratio of C1q binding material to Protein A binding material for five patient sera after passage over the HiPAC™ FPLC-CBP2 column.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

The C1q fragment of the invention may be any fragment of a C1q molecule, a plurality of which selectively binds immune complexes and aggregated immunoglobulins in the presence of monomeric immunoglobulin. The fragment may be a fragment of a C1q molecule of any species, such as human or rabbit. Suitable fragments may be identified as described in Example 1 below. The C1q fragment identified in Example 1 has the sequence

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln Ala Thr [SEQ ID NO 2]
1 5 10

and other suitable fragments may be identified by examining the amino acid sequences of C1q molecules for sequences homologous to this C1q fragment.

The invention also provides synthetic peptides comprising the sequence:

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln Ala Thr [SEQ ID NO 2]
1 5 10

or variants thereof capable of binding immunoglobulin. The synthetic peptides are also characterized in that a plurality of them selectively binds immune complexes and aggregated immunoglobulins in the presence of monomeric immunoglobulin.

The most preferred synthetic peptide has the sequence:

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln [SEQ ID NO 3]
1 5 10
Ala Thr Leu Leu Cys
15

The sequence

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln Ala Thr of SEQ ID NO 3 is that of the C1q fragment identified above (SEQ ID NO 2). The two leucine residues were added to the carboxyl terminus of this fragment as spacing residues to separate the potentially active residues from a future solid phase support (see below). The cysteine residue was added to the carboxyl terminus to allow for coupling of the peptide to solid phases. This preferred synthetic peptide is identified herein as CBP2.

As used herein, a "synthetic peptide" means a peptide which is not a naturally-occurring peptide, although "synthetic peptides" may be altered versions of naturally-occurring peptides. "Synthetic peptides" include peptides synthesized in vitro and peptides synthesized in vivo. In particular, "synthetic peptides" include peptides produced in transformed host cells by recombinant DNA techniques.

As used herein, "variant" means a synthetic peptide having changes (additions, deletions, or substitutions) in the specified amino acid sequence, provided that the "variant" synthetic peptide still has the ability to bind immunoglobulin. "Variants" can have a higher or lower affinity for immunoglobulin than the specified sequence, but all synthetic peptides according to the invention are characterized in that a plurality of them will selectively bind immune complexes and aggregated immunoglobulins in the presence of monomeric immunoglobulin. Preferred "variants" are those in which changes in the specified sequence are made so that the resulting sequence will assume an alpha helical structure when modeled by secondary structure prediction programs.

The C1q fragment or synthetic peptide may be made in a variety of ways. For instance, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield, in *Chem. Polypeptides*, pp. 335-61 (Katsoyannis and Panayotis eds. 1973); Merrifield, *J. Am. Chem. Soc.*, 85, 2149 (1963); Davis et al., *Biochem. Int'l*, 10, 394-414 (1985); Stewart and Young, *Solid Phase Peptide Synthesis* (1969); U.S. Pat. No. 3,941,763; Finn et al., in *The Proteins*, 3rd ed., vol. 2, pp. 105-253 (1976); and Erickson et al. in *The Proteins*, 3rd ed., vol. 2, pp. 257-527 (1976). Solid phase synthesis is the preferred technique of making individual C1q fragments and synthetic peptides since it is the most cost-effective method of making small peptides.

The C1q fragment and synthetic peptide may also be made in transformed host cells using recombinant DNA techniques. To do so, a recombinant DNA molecule coding for the fragment or peptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences coding for the C1q fragment could be excised from C1q genes using suitable restriction enzymes. Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidite method. Also, a combination of these techniques could be used.

The invention also includes a vector capable of expressing the C1q fragment or synthetic peptide in an appropriate host. The vector comprises the DNA molecule that codes for the C1q fragment or synthetic peptide operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation.

The vector must contain a promoter and a transcription termination signal, both operatively linked to the DNA molecule coding for the C1q fragment or synthetic peptide. The promoter may be any DNA sequence that shows transcriptional activity in the host cell and may be derived from genes encoding homologous or heterologous proteins (preferably homologous and either extracellular or intracellular proteins, such as amylases, glycoamylases, proteases, lipases, cellulases and glycolytic enzymes).

The promoter may be preceded by upstream activator and enhancer sequences. An operator sequence may also be included downstream of the promoter, if desired.

The vector should also have a translation start signal immediately preceding the DNA molecule, if the DNA molecule does not itself begin with such a start signal. There should be no stop signal between the start signal and the end of the DNA molecule coding for the C1q fragment or the synthetic peptide.

Expression control sequences suitable for use in the invention are well known. They include those of the *E. coli* lac system, the *E. coli* trp system, the TAC system and the TRC system; the major operator and promoter regions of bacteriophage lambda; the control region of filamentous single-stranded DNA phages; the expression control sequences of other bacteria; promoters derived from genes coding for *Saccharomyces cerevisiae* TPI, ADH, PGK and alpha-factor; promoters derived from genes coding for *Aspergillus oryzae* TKA amylase and *A. niger* glycoamylase, neutral alpha-amylase and acid stable alpha-amylase; promoters derived from genes coding for *Rhizomucor miehei* aspartic proteinase and lipase; and other sequences known to control the expression of genes of prokaryotic cells, eukaryotic cells, their viruses, or combinations thereof.

The vector must also contain one or more replication systems which allow it to replicate in the host cells. In particular, when the host is a yeast, the vector should contain the yeast 2u replication genes REP1-3 and origin of replication.

The vector should further include one or more restriction enzyme sites for inserting the DNA molecule coding for the C1q fragment or synthetic peptide and other DNA sequences into the vector, and a DNA sequence coding for a selectable or identifiable phenotypic trait which is manifested when the vector is present in the host cell ("a selection marker").

Suitable vectors for use in the invention are well known. They include pUC (such as pUC8 and pUC4K), pBR (such as pBR322 and pBR328), pUR (such as pUR288), phage λ and YEpl (such as YEpl24) plasmids and derivatives thereof.

In a preferred embodiment, a DNA sequence encoding a signal or signal-leader sequence, or a functional fragment thereof, is included in the vector between the translation start signal and the DNA molecule coding for the C1q fragment or synthetic peptide. A signal or signal-leader sequence is a sequence of amino acids at the amino terminus of a polypeptide or protein which provides for secretion of the protein or polypeptide from the cell in which it is produced. Many such signal and signal-leader sequences are known.

By including a DNA sequence encoding a signal or signal-leader amino acid sequence in the vectors of the invention, the C1q fragment or synthetic peptide encoded by the DNA molecule may be secreted from the cell in which it is produced. Preferably, the signal or signal-leader amino acid sequence is cleaved from the fragment or peptide during its secretion from the cell. If not, the fragment or peptide should preferably be cleaved from the signal or signal-leader amino acid sequence after its isolation.

Signal or signal-leader sequences suitable for use in the invention include *Saccharomyces cerevisiae* alpha factor (see U.S. Pat. No. 4,546,082), *S. cerevisiae* a factor (see U.S. Pat. No. 4,588,684), and signal sequences which are normally part of precursors of proteins or polypeptides such as the precursor of interferon (see U.S. Pat. No. 4,775,622).

The resulting vector having the DNA molecule thereon is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity to it of the C1q fragment or synthetic peptide encoded for by the DNA molecule, rate of transformation, ease of recovery of the C1q fragment or synthetic peptide, expression characteristics, bio-safety and costs. A balance of these

factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence.

Within these general guidelines, useful microbial hosts include bacteria (such as *E. coli* sp.), yeast (such as *Saccharomyces* sp.) and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

Next, the transformed host is cultured under conventional fermentation conditions so that the desired C1q fragment or synthetic peptide is expressed. Such fermentation conditions are well known in the art.

Finally, the C1q fragment or synthetic peptide is purified from the culture. These purification methods are also well known in the art.

The C1q fragment or synthetic peptide may be utilized to bind aggregated immunoglobulin or immune complexes. The C1q fragment or the synthetic peptide may be added directly to a fluid containing the aggregated immunoglobulin or immune complexes, or may be attached to a solid phase before being contacted with the fluid containing the aggregated immunoglobulin or immune complexes.

As noted above, a plurality of the C1q fragments or of the synthetic peptides selectively binds to immune complexes or aggregated immunoglobulins in the presence of monomeric immunoglobulin. This makes the C1q fragments and synthetic peptides particularly well-adapted to bind immune complexes and aggregated immunoglobulins in fluids containing monomeric immunoglobulin and for detecting or quantitating immune complexes in such fluids. Fluids which may contain immune complexes or aggregated immunoglobulins, as well as monomeric immunoglobulin, include body fluids (such as blood, plasma and serum) and reagent and pharmaceutical products (such as animal sera, solutions of gamma globulin, isolated blood components, solutions containing monoclonal antibodies, etc.).

Individual C1q fragments and synthetic peptides have a low affinity for immunoglobulins, including those in immune complexes and aggregated immunoglobulins. Accordingly, the plurality of C1q fragments or synthetic peptides must be held in sufficient proximity to each other so that multiple points of attachment to the immune complex or aggregated immunoglobulins can be made and a functional affinity formed (see the discussion of functional affinity in the Background section). This can be accomplished by forming oligomers of the C1q fragments or synthetic peptides (i.e., multiple copies of the C1q fragment or synthetic peptide on the same molecule) or by attaching the C1q fragments or synthetic peptides to a solid phase at an effective density.

On the oligomers, the C1q fragments and synthetic peptides will be spaced an adequate distance apart to permit the formation of multiple points of attachment with immune complexes and aggregated immunoglobulins. As a result, the oligomer will have a higher affinity for immune complexes and aggregated immunoglobulins than for monomeric immunoglobulin (i.e., a functional affinity). If necessary, amino acid spacers can be used between the C1q fragments and synthetic peptides to achieve the proper spacing. The oligomers can be prepared in the same ways as described above for the C1q fragment and synthetic peptide. The oligomers may also be attached to a solid phase as described below.

C1q fragments or synthetic peptides may also be attached to a solid phase at an effective density. An effective density is one that allows the immunoglobulin binding sites of the C1q fragments and synthetic peptides to be spaced apart on

the surface of the solid phase in such a manner as to permit multiple point attachment with the immune complexes or aggregated immunoglobulins. At this density, the C1q fragments and synthetic peptides will bind immune complexes and aggregated immunoglobulins in preference to monomeric immunoglobulin because a functional affinity develops. A density which is so low that the spacing of the C1q fragments or synthetic peptides exceeds the distance between binding sites on the immune complexes or aggregated immunoglobulins must be avoided. The density of C1q fragment or synthetic peptide which works best can be determined empirically and will depend on such factors as the surface area of the solid phase material, mode of coupling, the specific nature of the C1q fragment or synthetic peptide used, and the size of the immune complexes or aggregated immunoglobulins.

The C1q fragments, synthetic peptides and oligomers may be attached to any known solid phase material. For the C1q fragments and synthetic peptides, a solid phase which has a relatively non-porous surface is preferably used. Since the C1q fragments and synthetic peptides are small molecules, it is believed that they may become attached to the solid phase in the pores of a porous material. They may, therefore, bind immune complexes or aggregated immunoglobulins less readily since immune complexes and aggregated immunoglobulins are very large molecules which may not be able to enter the pores.

Suitable solid phase materials are well known in the art. Examples include silica, polyacrylamide, polymethylmethacrylate, polycarbonate, polyacrylonitrile, polypropylene, polystyrene, latex beads and nylon. Commercial sources of suitable solid phase materials include ChromatoChem (Missoula, Mont.), Pharmacia Fine Chemicals (Uppsala, Sweden), and others.

Also, the C1q fragment or synthetic peptide is preferably covalently attached to the solid phase material. Methods and agents for affecting this covalent attachment are well known in the art. Suitable agents include carbodiimide, cyanoborohydride, diimidoesters, periodate, alkylhalides, succinimides, dimethylpimelimidate and dimaleimides [See Blait, A. H., and Ghose, T. I., *J. Immunol. Methods*, 59: 129 (1983); Blair, A. H., and Ghose, T. I., *Cancer Res.*, 41: 2700 (1981); Gauthier, et al., *J. Exptl. Med.*, 156: 766-777 (1982)].

The C1q fragment or synthetic peptide is also preferably attached to the solid phase material by means of a spacer arm. The purpose of the spacer arm is to allow the fragment or peptide to be far enough away from the surface of the solid phase so that it can interact with the immune complexes and aggregated immunoglobulins which are very large molecules.

Suitable spacer arms include aliphatic chains which terminate in a functional group such as amine, carboxyl, thiol, hydroxyl, aldehyde, or maleimido, which is active in a coupling reaction. The spacer arm may be located on the solid phase or on the C1q fragment or synthetic peptide or, preferably, there is a spacer arm on both. If the spacer arm is located on the C1q fragment or synthetic peptide, it is preferably a peptide containing less than ten amino acids, preferably two to three amino acids.

The invention also comprises a method of detecting or quantitating immune complexes comprising contacting the immune complexes with the C1q fragment or the synthetic peptide so that the immune complexes bind to the fragment or the peptide. The C1q fragment or synthetic peptide may be added directly to fluids containing the immune complexes or may be attached to a solid phase of the types, and in the ways, described above.

The immune complexes can be detected or quantified using a labeled component that binds to the immune complexes or to the C1q fragment or synthetic peptide. For instance, labeled antibody to immunoglobulin could be used. The labels useful in the invention are those known in the art such as I^{125} , biotin, enzymes, fluorophores, bioluminescent labels and chemiluminescent labels. Methods of binding and detecting these labels are standard techniques known to those skilled in the art.

The immune complexes can be detected or quantitated using conventional immunoassay techniques. Such techniques include agglutination, radioimmunoassay, enzyme immunoassays and fluorescence assays. Enzyme immunoassays (EIA) are preferred since they provide a means for sensitive quantitation of levels of immune complexes. The specific concentrations, the temperature and time of incubation, as well as other assay conditions, can be varied in whatever immunoassay is employed depending on such factors as the concentration of the immune complexes or aggregated immunoglobulins in the sample, the nature of the sample and the like. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination while employing routine experimentation.

Since body fluids from mammals normally contain immune complexes, comparison of the levels of immune complexes in a test sample from a mammal will have to be made to the levels found in normals to identify levels of immune complexes indicative of a disease state.

A test kit for detecting or quantitating immune complexes is also part of the invention. The kit is a packaged combination of one or more containers holding reagents useful in performing the immunoassays of the invention.

The kit will comprise a container of the C1q fragment or a container of the synthetic peptide. The C1q fragment or synthetic peptide may be in solution or attached to a solid phase. The solid phases are the types described above, and the C1q fragment or synthetic peptide is attached as described above.

The kit may further comprise a container holding the above-described labeled component that reacts with either the immune complexes or the C1q fragment or synthetic peptide. Finally, the kit may also contain other materials which are known in the art and which may be desirable from a commercial and user standpoint. Such materials include buffers, enzyme substrates, diluents, standards, etc.

The C1q fragment or synthetic peptide can also be used as an anti-inflammatory drug. Inflammation in some diseases, such as rheumatoid arthritis, has been associated with the deposition of immune complexes in tissues and the activation of the complement cascade. It is the binding of C1q to immune complexes deposited in the tissues which initiates the complement cascade, and the action of the complement components alone or concurrently with other biological molecules, ultimately leads to tissue damage.

Accordingly, a C1q fragment or synthetic peptide according to the invention can be administered to a mammal suffering from inflammation mediated by the classical complement pathway to inhibit the binding of immune complexes by C1q and, thereby, prevent tissue damage and further inflammation. The C1q fragments or synthetic peptides are preferably injected at the site of inflammation in the mammal in order to obtain an adequate local concentration of C1q fragments or synthetic peptides. The advantage of such a therapeutic approach is that small peptides are less likely to illicit an immune response which would render the drug inactive.

Finally, the invention provides a binding material for removing immune complexes or aggregated immunoglobu-

lins from a fluid. The material comprises plural binding peptides, the peptides being characterized in that a plurality of them selectively binds immune complexes or aggregated immunoglobulins in the presence of monomeric immunoglobulin. Preferably, the binding peptides are the C1q fragments of the invention or synthetic peptides comprising the sequence

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln Ala Thr [SEQ ID NO 2]
1 5 10

or combinations of the two.

Individual binding peptides have a low affinity for immunoglobulin. Accordingly, the plurality of binding peptides must be held in sufficient proximity to each other so that multiple points of attachment to the immune complex or aggregated immunoglobulins can be made and a functional affinity is formed. This can be accomplished by forming oligomers of the binding peptides or by attaching the binding peptides to a solid phase at an effective density as described above for the C1q fragments and synthetic peptides. The methods of preparation, and properties, of the resulting oligomers and solid phase materials are as described above for the C1q fragments and synthetic peptides. Also, the oligomers of C1q fragments or synthetic peptides and the solid phase materials having C1q fragments or synthetic peptides attached to them at an effective density that are described above are examples of binding materials according to the invention.

Immune complexes and aggregated immunoglobulins can be removed from fluids containing them by contacting the fluids with the binding material, and then separating the fluid from the binding material. The fluid is simply contacted with the binding material. Such contact can be effected by passing the fluid through a device containing the binding material so that the fluid may contact the binding material. Alternatively, the fluid may be incubated statically with the binding material in the device. The duration of the contact is not bound to critical limits although it should, of course, be sufficient to allow aggregated immunoglobulin or immune complexes to be bound by the binding peptides. The binding material of the invention is especially well-adapted to be used to remove aggregated immunoglobulins or immune complexes from fluids containing monomeric immunoglobulin such as those listed above.

The invention also comprises a device for removing immune complexes or aggregated immunoglobulins from a fluid. The device comprises the binding material and a means for encasing the material so that the fluid can be contacted with it. Binding materials having binding peptides attached to a solid phase are preferred.

The encasing means may be a plastic bag, a column, a test tube, plastic tubing, encasing means like those used on plasmapheresis devices, and other suitable encasing means. The encasing means should be made of a material which is not harmful to the fluid to be placed in the device.

Thus, the device may be a typical plasmapheresis device in which the solid phase is a membranous surface or hollow fibers to which the binding peptides are attached. The device may also be a column packed with beads or any suitable solid phase having the binding peptides attached to it. The device may be a test tube filled with beads to which the binding peptide is attached.

Other devices are also possible. For instance, the binding material may be beads (such as silica beads) to which the binding peptides are attached at a suitable density to selectively remove immune complexes or aggregated immunoglobulins from fluids containing monomeric immunoglobulin.

lin. The means for encasing the beads may be a plastic bag of e.g., the type used for transfusions. The fluid containing the immune complexes or aggregated immunoglobulins is mixed with the beads in the bag and incubated for a time, and at a temperature, sufficient to allow the immune complexes or aggregated immunoglobulins to bind to the binding peptides. Then the beads are allowed to settle (or may be centrifuged), and the fluid, from which immune complexes have been removed, is decanted.

For therapeutic uses, a solid phase binding material can be encased online in an extracorporeal device through which whole blood or plasma can be circulated dynamically so that the immune complexes contained therein are bound and removed from the blood or plasma. An alternative would be to statically incubate the whole blood or plasma in a device such as the plastic bag device described above. In either case, fluids can be returned to the body after the incubation or passage is complete, negating the need for blood replacement therapy.

A device intended for therapeutic use may also include appropriate tubing for connecting it to a patient and a pump to aid the passage of the fluid through the device and back into the patient and to prevent air from entering the system. The device must be sterilized for therapeutic use, and sterilization may be accomplished in conventional ways such as purging with ethylene oxide or by irradiating the device.

EXAMPLES

Unless otherwise indicated, the chemicals used in the following Examples were obtained from Sigma Chemical Co., St. Louis, Mo.

Example 1

Peptide Synthesis

A. Peptide Design and Structure

X-ray crystal structural data for Protein A Fragment B indicated that two coplanar α -helical segments with the proper contact residues (see Background) could bind immunoglobulin Fc with a high affinity. Deisenhofer et al., *Hoppe-Seyler's Z. Physiol. Chem. Bd.*, 359, S. 975-85 (1978); Burton, *Molec. Immunol.*, 25, 1175-81 (1988); Langone, *Adv. Immunol.*, 32, 157-252 (1982). As discussed above, C1q also binds IgG Fc. Thus, the question was posed whether C1q bound IgG Fc using structures similar to those found in Protein A Fragment B. Unfortunately, structural information regarding the C1q molecule was limited, and information concerning the C1q binding site for immunoglobulin was nonexistent.

However, the amino acid sequences of the entire A and B chains and a partial sequence for the C chain of C1q were available. Reid, *Biochemical J.*, 179, 367-71 (1978); Reid, et al., *Biochemical J.*, 203, 559-69 (1982). These sequences were analyzed by the secondary structure prediction programs of Garnier et al., *J. Molec. Biol.*, 120, 97-120 (1978) and Chou and Fasman, *Adv. Enzym.*, 47, 45-146 (1978). Of the predicted alpha helical regions, one helical region in the C1q B chain globular head region spanned 12 residues, suggesting a high probability that the helix prediction was accurate.

The predicted helical segment of C1q B chain (residues 189-200) was then compared to one Protein A Fragment B helix (residues 142-153), and homology was observed (see FIG. 1). Four amino acids were exact matches, while three additional residues showed conservative substitutions. Out

of three possible contact residues on the Protein A helix. C1q possessed two similar residues. Further analysis of the two sequences was achieved through the use of helical wheel diagrams (FIG. 2). These diagrams showed that the pattern of hydrophobic and hydrophilic residues observed for the Protein A helix known to contact immunoglobulin was similar to that of the predicted C1q helix.

Therefore, the 12-residue sequence of C1q having the predicted helical structure was synthesized. Two Leu residues were added to the carboxyl-terminus as spacing residues to separate the potentially active residues from a future solid phase matrix. A Cys residue was also added to the carboxyl-terminus to allow for coupling to solid phases using certain coupling procedures. The final amino acid sequence, designated CBP2, is presented in FIG. 1.

B. Synthesis

The CBP2 sequence was submitted to Biosearch Inc. (San Raphael, Calif.) for custom peptide synthesis using the standard Merrifield solid phase synthesis approach. Merrifield, in *Chem. Polypeptides*, pp. 335-61 (Katsoyannis and Panayotis eds., Plenum, New York, N.Y. 1973). Approximately 250 mg of the peptide in lyophilized form were obtained.

Biosearch's quality control information was based on amino acid analysis and analytical reverse phase HPLC. The chromatograph provided by Biosearch showed that the initial purity of the peptide was approximately 40-50%. Biosearch did not provide the results of the amino acid analysis, but they stated that the composition of the peptide had to be within $\pm 20\%$ of the calculated values for each amino acid of the peptide in order to pass their quality control. Therefore, the CBP2 peptide should have had the correct amino acid sequence.

C. Purification

In addition to the purification performed by Biosearch, the peptide was further purified by gel filtration chromatography. The lyophilized CBP2 was not soluble in aqueous solvents and required 50% (v/v) N,N-dimethylformamide (DMF) (J. T. Baker Chemicals, Phillipsburg, N.J.) to completely dissolve. Due to the high hydrophobic character of the solvent, Sephadex G25 (Pharmacia, Piscataway, N.J.) was used for the chromatography. A column of dimensions 0.7x40 cm was poured and equilibrated with 1% (v/v) DMF in distilled water (dH₂O). A 2 mg/ml sample of CBP2 was prepared in 50% (v/v) DMF, and 1 ml of the sample was loaded on the column and eluted with 1% DMF at a flow rate of 1 ml/15 min. One ml fractions were collected.

The column fractions were assayed for peptide by the Bicinchoninic Acid (BCA) protein assay (Pierce Chemical Co., Rockford, Ill.) according to the manufacturer's instructions. Briefly, duplicate 30 μ l aliquots from each fraction were placed in the wells of a microtiter plate, followed by the addition of 300 μ l of BCA protein reagent. In addition to the column fractions, samples ranging from 1 mg/ml to 0.1 μ g/ml Val-Gly-Gly peptide (Sigma Chemical Co., St. Louis, Mo.) were also added to the microtiter plate to prepare a standard curve. The plate was incubated at 37° C. for 1 hour, and the resultant colored product measured using a TiterTek Multiskan plate reader (Flow Laboratories, McLean, Va.) at 560 nm. The absorbances were then plotted against fraction number to generate the chromatogram.

A 1% (v/v) DMF sample was also tested in the BCA assay as a control. DMF was shown by these experiments not to interfere with the detection of the peptides in the column fractions.

A 1.2 fold increase in peptide purity with a 41% yield of peptide was obtained. A representative chromatograph for

CBP2 is shown in FIG. 3. The profile showed a symmetrical major peak at 250 minutes and several minor peaks representing contaminants. It should also be noted that purified CBP2 was readily soluble in 1% (v/v) DMF, indicating that it may be the impurities in lyophilized CBP2 that could only be dissolved in 50% DMF.

In addition to the standard BCA assay for peptides, the absorbance profile for CBP2 migrating in the Sephadex G25 column was measured at 230 nm. The absorbance profile shown in FIG. 3 confirms the results of the BCA assay in that the CBP2 peak had a retention time of 250 minutes. It is important to note that the UV absorbance profile at 230 nm is most sensitive to amide bonds. Since the CBP2 peak was the major source of absorbing material, the remaining impurities may not contain amide bonds and, therefore, may not be peptides.

The CBP2 retention time in the Sephadex G25 column suggested a larger molecular weight than the calculated molecular weight based on the amino acid sequence of the peptide. Therefore, under the same conditions, samples of glucagon, LHRH, and a mixture of glucagon and LHRH were loaded and eluted from the Sephadex G25 column. Glucagon (3550 daltons) had a retention time of 247 minutes and LHRH (1182.33 daltons) had a retention time of 277.4 minutes. CBP2 (1556.98 daltons) had a retention time similar to glucagon, suggesting that CBP2 migrated through the Sephadex G25 column as an aggregate of at least two peptides. Concentration dependent aggregation of amphipathic peptides has been documented. DeGrado et al., *J. Am. Chem. Soc.*, 103, 679-81 (1981); Taylor et al., *Mol. Pharmacol.*, 22, 657-66 (1982); Moe and Kaiser, *Biochem.*, 24, 1971-76 (1985).

Analytical C₁₈ reverse phase HPLC was used in order to assess the purity of the CBP2 isolated from the Sephadex G25 column. The conditions used for the chromatography were the same as those used by Biosearch. Briefly, 500 μ g/ml of CBP2 in 1% (v/v) DMF in dH₂O and 4 mg/ml dithiothreitol (the peptide was incubated over night with the DTT in order to reduce any disulfide bonds that may have formed) were passed over a Vydac (Hesperia, Calif.) C₁₈ analytical column (product #218TP54) with dimensions 4.6 mmx25 cm and using the following buffers and conditions. Buffer A: 0.05% (v/v) trifluoroacetic acid (TFA) in dH₂O. Buffer B: 0.05% (v/v) TFA in acetonitrile. Column conditions: A 100 μ l sample was injected, and 5% buffer B was maintained for 3 min at 1.7 ml/min followed with a gradient of 5-100% buffer B over the next 20 min. The peptide was detected with a Beckman (Palo Alto, Calif.) UV variable wavelength detector at 230 nm.

It is important to note that CBP2 was treated with DTT overnight before the analysis in order to disrupt any disulfide bonds that may have formed. FIG. 4A shows a representative chromatograph of the reduced isolated CBP2. CBP2 comprised 80% of the absorbing material, indicating that the Sephadex G25 isolated CBP2 was relatively pure. With exception of the peaks at 4.4 minutes, the remaining peaks were retained in the column significantly longer than CBP2, suggesting that the impurities were of smaller molecular weight and/or were much more hydrophobic than CBP2.

Since CBP2 did contain free cysteine residues, it was necessary to determine the CBP2 profile in the absence of DTT to assess the extent of disulfide formation. In addition, K₃Fe(CN)₆, which would favor the formation of disulfides, was added to the reduced CBP2 solution. This process was necessary to determine the retention time of the disulfide aggregated CBP2. FIG. 4B shows the CBP2 profile without the reduction of disulfides. A strong absorbance peak at 4.4

minutes possessing 28% of the total absorbance was the only significant difference between reduced and nonreduced CBP2 (compare FIGS. 4A and 4B). The addition of $K_3Fe(CN)_6$ to the reduced CBP2 solution and the subsequent analysis showed that the peak at 4.4 minutes in FIG. 4B was due to disulfide aggregated CBP2. In FIG. 4C, the addition of the oxidizing reagent caused a decrease in the reduced CBP2 peak with an accompanying increase in the 4.4 minute peak which represented 68.7% of the total area. Therefore, the 4.4 minute peak was the disulfide aggregated CBP2.

It is important to note that in all three chromatographs in FIG. 4, CBP2 comprises at least 80% of the total area, with the combination of the reduced and nonreduced CBP2 peaks representing approximately 90% of the total area. Based on the results of the three chromatographs, it was concluded that the purity of CBP2 was 80-90%.

D. CD Spectroscopic Analysis Of CBP2

Circular dichroism (CD) spectroscopy was performed on an Aviv 60DS spectropolarimeter. The sample consisted of 200 μ l of 200 μ g/ml CBP2 dissolved in 10 mM sodium phosphate buffer, pH 7.0, at room temperature. A 1 mm path length was used.

Under these conditions, CBP2 exhibited a CD spectrum characteristic of a random coil peptide. The lack of structure was expected for small peptides such as CBP2.

Example 2

Inhibition Studies

A. Titration of Rabbit Immunoglobulin Bound by Solid Phase Protein A

Protein A (recombinant Protein A from Repligen, Cambridge, Mass.) diluted to 1 μ g/ml in coating buffer (0.1M $NaHCO_3$, pH 9.0) was incubated in the wells of a microtiter plate (100 μ l per well) for 2 hours at 37° C. Uncoated surfaces of the wells were blocked by adding 340 μ l PBSC (9 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , pH 7.2, 154 mM NaCl, 1 mg/ml casein or ovalbumin, and 0.01% thimerosal), incubating the plate 1 hour at 37° C., and then washing the wells 3 times with PBSC (PBSC plus 0.1% Tween 20). Dilutions of rabbit immunoglobulin labeled with horseradish peroxidase (Ig-HRP) (Zymed Laboratories, Inc., South San Francisco, Calif.) in PBS (9 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , pH 7.2, 154 mM NaCl) were incubated in the wells (100 μ l per well) for 2 hours at 37° C. followed by washing 3 times with PBS. OPD substrate buffer (0.8 mg/ml o-phenylenediamine, 0.1M Na_2HPO_4 , 0.05M sodium citrate, pH 5.5, plus 0.15% (v/v) H_2O_2) was then added to the wells (100 μ l/well), and the resultant colored product was measured with a Titertek Multiskan plate reader using a 414 nm filter.

CBP2 Inhibition of Solid Phase Adsorbed Protein A.

It had previously been reported that C1q and Protein A compete exclusively for IgG (Burton, *Molec. Immunol.*, 22, 161-206 (1985); Stalinhelm et al., *Immunochem.*, 10, 501-507 (1973); Langone et al., *J. Immunol.*, 121, 327-38 (1978); Lancet et al., *Biochem. Biophys. Res. Commun.*, 85, 608-614 (1978)). Accordingly, CBP2 was tested for inhibition of the binding of Ig-HRP by Protein A.

The wells of a microtiter plate were coated with Protein A as described above in Part A. Ig-HRP diluted 1:5000 in PBS (the dilution determined by the protein A titration experiment of Part A; original Ig-HRP concentration of 3.3 mg/ml) was preincubated with various concentrations of CBP2 for 1 hour at 37° C. before 100 μ l of the incubation mixture were added to each well of the microtiter plate. The CBP2/Ig-HRP mixture was incubated with the solid phase

adsorbed Protein A for 2 hours at 37° C. and then washed 3 times with PBS. OPD substrate buffer was then added (100 μ l/well), and the resulting colored product was measured at 414 nm in a Titertek Multiskan plate reader. Absorbance readings were made when the control wells with no inhibitor had an absorbance of 1.0 or greater.

The results, which are shown in FIG. 5, show that CBP2 was able to inhibit Protein A binding of Ig-HRP by nearly 100%. The concentration at 50% inhibition was approximately 1 μ M which is a 1000-fold greater concentration than the 1 nM 50% inhibition concentration for solution phase Protein A (see FIG. 5). Since the 50% inhibition concentration for Protein A is comparable to its dissociation constant, the 50% inhibition concentrations were considered a good measure of the binding affinity of the inhibitor.

C. Titration of Ig-HRP Bound by Solid Phase C1q.

C1q (gift of Dr. Lawrence Potempa, Immtech International Inc., Evanston, Ill.) diluted to 10 μ g/ml in coating buffer was placed in the wells of a microtiter plate (100 μ l/well) and incubated for 2 hours at 25° C. The uncoated surfaces of the wells were blocked by adding 340 μ l PBSC and incubating the plate for 1 hour at 25° C., followed by washing 3 times with PBSC. Dilutions of Ig-HRP in C1q buffer (50 mM Tris, pH 7.2, 27 mM NaCl, 1 mM $CaCl_2$, 0.01% (wt/v) Thimerosal) were incubated in the wells (100 μ l/well) for 1 hour at 25° C. followed by washing 3 times with C1q buffer. OPD substrate buffer was then added (100 μ l/well), and the colored product was measured at 414 nm in a Titertek Multiskan plate reader.

D. CBP2 Inhibition of Solid Phase C1q.

C1q was coated onto the surface of the wells of a microtiter plate as described in Part C. Ig-HRP diluted 1:100 (the dilution determined from the C1q titration experiment described above in Part C) in C1q buffer was preincubated with various concentrations of CBP2 for 1 hour at 25° C. The Ig-HRP/CBP2 mixture was then added to the microtiter plate (100 μ l/well), and the plate was incubated for 1 hour at 25° C., followed by 3 washes with C1q buffer. OPD substrate buffer (100 μ l/well) was then added and the colored product measured at 414 nm in a Titertek Multiskan plate reader.

The resulting inhibition curve is presented in FIG. 6. The 50% inhibition concentration was approximately 10 μ M, which was an order of magnitude higher than the 50% inhibition concentration for CBP2 inhibiting Protein A. The 50% inhibition concentration for liquid phase C1q inhibiting solid phase C1q was approximately 10 nM or 1000 fold less than the CBP2 concentration (see FIG. 6).

E. Control Experiments For CBP2 Inhibition Of Protein A And C1q

In order to conclude that CBP2 was binding immunoglobulin and not causing an inhibition in the assay system through a nonspecific interaction, a series of control experiments were performed.

1. The effects of CBP2 on the HRP enzyme were investigated. Ig-HRP at a 1:100 dilution in PBS or C1q buffer was incubated with 50 μ M CBP2 or an equal amount of 1% (v/v) DMF for 3 hours at 37° C. Aliquots of 100 μ l were placed in the wells of a microtiter plate and an equal volume of OPD substrate buffer was added, and the colored product was measured at 414 nm. Both the CBP2 and Ig-HRP were at the highest concentrations tested in the inhibition assay. The sample which contained both peptide and Ig-HRP did not differ significantly from the control sample containing the Ig-HRP alone. Therefore CBP2 did not inhibit the HRP enzyme.

2. Protein A at 1 μ g/ml or C1q at 10 μ g/ml diluted in coating buffer was incubated in the wells of a microtiter

plate (100 μ l/well) for 2 hours at 37° C. or 25° C., respectively. The wells were blocked in the same fashion as described above for each assay. Various concentrations of CBP2 diluted in PBS or C1q buffer were incubated in the wells (100 μ l/well) for 2 hours at 37° C. or 1 hour at 25° C., respectively, followed by washing the wells 3 times with either PBS or C1q buffer. Ig-HRP was then added at the appropriate dilution in either PBS or C1q buffer and incubated under the appropriate conditions, followed by washing 3 times with PBS or C1q buffer. OPD substrate buffer was then added (100 μ l/well), and the colored product was measured at 414 nm as described above.

3. Excess protein (1 mg/ml casein) was added to the buffers used to dilute the Ig-HRP/CBP mixture.

Controls 2 and 3 tested for nonspecific protein-protein or protein-peptide interactions. If there were such interactions, then there would be a loss of CBP2's inhibitory activity in test 3, and no such loss was observed. In test 2, any loss in CBP2's inhibitory activity would indicate that CBP2 was blocking the solid phase Protein A or C1q binding sites and not interacting with Ig-HRP. No loss of inhibitory activity was detected.

4. Equal molar concentrations of luteinizing hormone releasing hormone (LHRH) (Beckman Instruments, Palo Alto, Calif.) or various concentrations of Protein A were substituted for CBP2 in the inhibition assay described in Part B. In the case of the C1q inhibition experiments, LHRH and C1q were substituted for CBP2 as a negative and a positive control, respectively. The use of LHRH also tested whether the inhibition of Protein A or C1q by CBP2 was due to CBP2 specifically, or simply due to nonspecific effects attributable to a small peptide. The results are shown in FIGS. 5 and 6. As indicated in these figures, LHRH demonstrated no Protein A or C1q inhibitory activity over the same concentration range examined for CBP2, and both Protein A and C1q were inhibitory, as expected.

5. Concentrations of Ig-HRP required for the C1q assays were 50 fold higher than the concentration used for Protein A assays. The reason for the higher concentration is that only a small fraction of Ig-HRP existed as complexes that could be bound by C1q. This was demonstrated by fractionating the Ig-HRP as follows. A 40 μ l sample of Ig-HRP was loaded onto a TSK SW 4000 sizing HPLC column (Beckman/Altex TSK 4000 SW, 7.5 mm \times 30 cm). Only the high molecular mass eluted fractions exhibited any C1q binding activity as determined by a C1q binding assay performed as described above. The results of this assay indicated that the C1q binding material was a fraction having molecular weight of approximately 1,000 kdal, indicating a complex of immunoglobulins and HRP enzymes, and not a monomeric immunoglobulin plus HRP enzyme.

6. With respect to C1q, nonspecific ionic interactions could not be excluded since the C1q buffer was half-physiological ionic strength. The ionic strength of the C1q buffer could not be changed since C1q binding of immunoglobulins is partially mediated by ionic interactions. However, based on the Protein A inhibitory activity of CBP2 in physiological ionic strengths, it was a reasonable assumption that CBP2 inhibition was not primarily the result of nonspecific ionic interactions.

7. Proteins when adsorbed to the polystyrene wells of a microtiter plate may partially lose their native conformation. Therefore, CBP2 was also screened for any interactions with solution phase Protein A. Protein A was incubated in the wells of a microtiter plate (100 μ l/well) for 2 hours at 37° C. at 1 μ g/ml in coating buffer, and the wells blocked as described above. Protein A at a constant concentration

(corresponding to either 100 times the 50% inhibition concentration, the 50% inhibition concentration, or 10 fold less than the 50% inhibition concentration) was preincubated with CBP2 and Ig-HRP in PBS for 1 hour at 37° C. The Protein A/CBP2/Ig-HRP mixture was then incubated in the wells of the microtiter plate (100 μ l/well) for 2 hours at 37° C. and washed 3 times with PBS. OPD substrate buffer was then added (100 μ l/well), and the resultant colored product was measured at 414 nm as before.

At Protein A concentrations at or above the 50% inhibition concentration, there was a linear, additive response, indicating no interaction between CBP2 and Protein A. At Protein A concentrations below the 50% inhibition concentration, the curve is analogous to the CBP2 inhibition curve in the absence of solution phase Protein A. This also suggests that CBP2 and Protein A are not interacting.

8. Since the CBP2 peptide has a free cysteine residue, the effects of DTT on the inhibition assay were examined. DTT was added to the buffer containing CBP2 (final DTT concentration 4 mg/ml), and the CBP2 was incubated with the DTT for 2 hours at 37° C. to ensure that the cysteine residues of CBP2 were reduced. Then the inhibition assay using solid phase Protein A was performed as described above.

The addition of DTT to the CBP2 solution had no effect on the inhibitory activity of CBP2. These data indicate that CBP2 does not inhibit by forming disulfide interaction with Protein A or Ig-HRP and that disulfide-mediated CBP2 aggregates did not have a significant influence on CBP2's ability to inhibit the binding of Ig-HRP to Protein A.

F. Conclusions

Based on the inhibition data in conjunction with the above controls, it can be concluded that CBP2 inhibits C1q and Protein A binding of Ig-HRP through a binding interaction with solution phase Ig-HRP. The results indicate that CBP2 binds immunoglobulin in a specific manner.

Example 3

HiPACT™ LTQ Column

A HiPACT™ LTQ (ChromatoChem, Missoula, Mont.) activated aldehyde column of dimensions 7.4 mm \times 1.9 cm was equilibrated with 6 ml of immobilization buffer (0.1M sodium citrate, pH 5.5). This column is composed of silica beads to which a long carbon chain spacer arm is attached. A ligand coupling solution containing 500 μ g CBP2 and 20 mg/ml sodium cyanoborohydride in immobilization buffer was prepared. This was continuously circulated through the column for 5 min at 25° C. at a flow rate of 1–2 ml/min. The coupling solution was then eluted from the column and collected. The column was subsequently washed with 6 ml immobilization buffer which was also collected. The column was then washed with 6 ml of 2% (v/v) acetic acid and equilibrated with 6 ml C1q buffer.

The coupling procedure linked CBP2 to the solid phase support through the N-terminal amino group, leaving the C-terminal cysteine's sulfhydryl available as a marker. The Ellman's reagent assay (Ellman, *Arch. Biochem. Biophys.*, 82, 70–77 (1959)) was, therefore, used to measure the amount of CBP2 bound to the column since Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid)) reacts with free sulfhydryl groups.

To perform the Ellman's reagent assay, the column was first washed with a 4 mg/ml solution of DTT in order remove any peptides linked to the solid phase by disulfide bonds. Then the column was washed with C1q buffer until the eluate showed no reactivity with Ellman's reagent. The column was then equilibrated with 6 ml of 0.1N Na₂HPO₄.

pH 8.0. Next, 1 ml of a solution of 400 $\mu\text{g/ml}$ Ellman's reagent in 0.1N Na_2HPO_4 , pH 8.0, was continuously passed over the column for 15 min. The Ellman's reagent solution was eluted from the column, collected, and the column washed with 6 ml of 0.1N Na_2HPO_4 , pH 8.0. One ml of a 4 mg/ml solution of DTT was passed over the column, and the eluate was collected. The column was then washed with 6 ml C1q buffer and the eluates collected. The resulting colored products were measured at 412 nm.

The moles of CBP2 coupled to the column were proportional to the moles of Ellman's reagent reacting with the column (i.e., the amount of Ellman's reagent eluted with the DTT) which was calculated using a molar extinction coefficient of $1.36 \times 10^4/\text{cm} \cdot \text{M}$ for the free thionitrobenzoic acid. The assay of the column indicated that 84% of the CBP2 added to the column was coupled to the solid phase, or approximately 420 μg CBP2.

It should be noted that attempts to measure CBP2 in eluted fractions using various techniques (BCA assay, UV spectroscopy, 2,4,6-trinitrobenzene sulfonic acid and the Ellman's reagent assay) were unsuccessful because the cyanohydride reagent interfered with those assays.

Two control columns were prepared along with the CBP2 column. LHRH was coupled to the matrix in the same fashion as CBP2 and at the same concentration as CBP2 as determined by UV spectroscopy. A similar percentage (78%) of LHRH was coupled to the column by reductive amination as with CBP2 (84%). Another column was treated in the same fashion as the CBP2 and LHRH columns, but no peptide or protein was coupled ("No peptide" column), thus allowing the study of nonspecific binding effects in the following experiments.

Example 4

PAP Binding to the HIPACTTM LTQ-CBP2 Column

A CBP2 column prepared as described in Example 3 was equilibrated with 3 ml C1q buffer. Immune complexes composed of rabbit anti-horseradish peroxidase and horseradish peroxidase (PAP) (purchased from Organon Teknica/Cappel, West Chester, Pa.) at a concentration of 20 $\mu\text{g/ml}$ in C1q buffer (a total volume of 1 ml) were loaded onto the column and incubated for 15 min at 25° C. with continuous circulation through the column. Unbound PAP complexes were eluted with 12 ml C1q buffer (at a flow rate of 0.33 ml/min, collecting 1 ml fractions) followed by 3 ml of 2% (v/v) acetic acid to elute bound PAP.

A. Peroxidase Assays

The column fractions were assayed for peroxidase activity by placing duplicate 20 μl aliquots into the wells of a microtiter plate and adding 100 μl of OPD substrate buffer. The absorbances were measured at 414 nm in a Titertek Multiskan plate reader. The absorbances were then plotted against fraction number to generate the chromatograph. In addition to the samples, a standard curve was prepared, and a linear regression analysis was performed. From the standard curve the concentration of PAP in each fraction was calculated.

The resulting profile is presented in FIG. 7. The elution profile shows two major peaks. The first peak was material that did not bind to the column and which was readily eluted with C1q buffer. This material appeared to make up the larger fraction of the PAP loaded on the column. In fact, using the standard curve and linear regression analysis, the flow-through peak was calculated to contain approximated 90-95% of the total PAP. The second peak was the material

bound by the column and eluted with the acetic acid. The bound material was only 5-10% of the total PAP loaded on the column.

Although the efficiency of the column appeared to be low, the column did bind immune complexes. Therefore, it was determined whether the column bound immune complexes specifically and in a CBP2-dependent manner.

One ml containing PAP complexes at a concentration of 20 $\mu\text{g/ml}$ and monomeric rabbit IgG at a concentration of 80 $\mu\text{g/ml}$ in C1q buffer was loaded on a CBP2 column prepared as described in Example 3, that had been equilibrated with 3 ml of C1q buffer. The PAP/IgG mixture was circulated through the column continuously for 15 min and then eluted with 12 ml C1q buffer at a flow rate of 0.33 ml/min. Material bound to the column was eluted with 3 ml of 2% (v/v) acetic acid followed by 6 ml of C1q buffer. (The flow rates and fractions were the same as for PAP alone.) The fractions were assayed for the presence of peroxidase activity in the same fashion as described above and the concentration of PAP was calculated from the standard curve.

Once again two peaks were observed (FIG. 8). The first peak represented the flow-through, and contained 95% of the total PAP loaded on the column. The amount of material bound to the column in the second peak also remained unchanged at approximately 5%. Since the elution profiles of PAP, both with and without monomeric IgG, were not different with respect to the quantities of material in each peak and the retention times of the peaks, it appeared that the column specifically bound immune complexes.

B. Enzyme Immunoassay For IgG

PAP complexes were loaded onto a CBP2 column and eluted from it as described in Part A. Also, monomeric IgG at a concentration of 80 $\mu\text{g/ml}$ in 1 ml of C1q buffer was loaded onto a CBP2 column and eluted from it in the same manner.

The fractions were then assayed for immunoglobulin by enzyme immunoassay as follows. Duplicate 50 μl aliquots of each fraction were placed in the wells of a microtiter plate. Fifty μl of coating buffer were then added, and the plate was incubated at 37° C. for 2 hours. The uncoated surfaces of the wells were then blocked by adding 340 μl of PBSC, followed by incubation of the plate for 1 hour at 37° C. The wells were washed 3 times with PBSCT, and a 1:2000 dilution of goat anti-rabbit IgG that was labeled with biotin (anti-rabbit IgG) (Vector Laboratories, Inc., Burlingame, Calif.) was added (100 $\mu\text{l}/\text{well}$). The anti-rabbit IgG was incubated in the wells for 2 hours at 25° C., and the wells were washed 3 times with PBSCT. Next, a 1:2000 dilution of streptavidin- β -galactosidase (BRL-Life Technologies, Gaithersburg, Md.) in PBSCT was added to the wells (100 $\mu\text{l}/\text{well}$), the plates were incubated for 2 hours at 25° C. and washed 3 times with PBSCT. Fluorogenic substrate in buffer (5 mg/ml of 4-methylumbelliferyl- β -D-galactoside in DMF diluted, 1:50 in 0.01M sodium phosphate buffer, pH 7.5, containing 0.1M NaCl and 1 mM MgCl_2) was added to the wells (100 $\mu\text{l}/\text{well}$), and the resulting fluorescence was measured with a Dynatech MicroFluor plate reader (Dynatech, Alexandria, Va.) using 365 nm as the excitation wavelength and 450 nm as the emission wavelength.

In addition to the samples, standard curves of IgG and PAP dilutions were prepared, and a linear regression analysis was performed. The standards demonstrated a linear response in fluorescence to PAP and IgG concentration as confirmed by the linear regression analysis. From the standard curves, the concentration of PAP or IgG in each fraction was calculated.

The linear regression analysis showed that 44% of the PAP loaded on the column were bound to the column. The

amount of monomeric IgG bound to the column was 4.4% of the total IgG loaded on the column. See Table 1 below. These results show that the column was not binding a significant amount of monomeric IgG and was specifically binding immune complexes.

Clearly, there was a large discrepancy between the results of the peroxidase assay (about 5% binding of immune complexes) and the enzyme immunoassay for immunoglobulin (about 44% binding of immune complexes). Closer examination of the kinetics of the two enzymatic reactions revealed that the peroxidase assay reached its maximal absorbance in 5-10 minutes on average, while the streptavidin- β -galactosidase assay reached its maximal fluorescence in 25-30 minutes. Therefore, although the two assays both gave linear responses to incremental increases in concentration of PAP, the enzyme immunoassay measured the relative quantities of PAP more accurately and sensitively due to the longer incubation time.

As a result of the comparison of the results of the peroxidase assay and enzyme immunoassay, elution profiles were reassessed using the enzyme immunoassay. Also, PAP complexes were loaded onto and eluted from the control LHRH and No peptide columns in the same manner as described above for the CBP2 column and assayed using the enzyme immunoassay. The results are shown in Table 1 below.

TABLE 1

Sample	Percent Bound to Column		
	CBP2 Column	LHRH Column	No Peptide Column
PAP	44	4.8	8
Monomeric Rabbit IgG	4.4	N.D.*	N.D.*

*Not Determined

The immune complex binding efficiency of the CBP2 column as shown in Table 1 was much higher than had been previously calculated using the peroxidase assay. The results also indicated that the column specifically bound immune complexes. There was very little binding of the immune complexes to the control LHRH and No peptide columns, showing that the binding of immune complexes was not due to nonspecific binding effects.

C. Elution With CBP2

If PAP binding was a specific interaction between the solid phase CBP2 and the immunoglobulins of PAP, then PAP bound by the column should be eluted using a solution of CBP2, and a solution of a control peptide (LHRH) should cause no elution of bound PAP. This was exactly what was found by the following experiment.

A 1 ml sample of 20 μ g/ml PAP was loaded on an equilibrated CBP2 column (prepared as described in Example 3) and allowed to circulate continuously for 15 min over the column. The column was then washed with 12 ml C1q buffer, followed by 3 ml of 336.2 μ g/ml CBP2 (this CBP2 concentration was 20 times the concentration of CBP2 needed to give 50% inhibition Ig-HRP binding to C1q) diluted in C1q buffer. An additional 6 ml of C1q buffer was used to wash the column followed by 3 ml of 2% (v/v) acetic acid and then with 6 ml of C1q buffer to completely wash the column. Fractions were collected and assayed for the presence of peroxidase activity as described above in Part A. A control column was run as described above, except that 3 ml of LHRH at an equimolar concentration as the CBP2 solution, was substituted for the CBP2 wash.

As shown in FIG. 9A, the normal flow-through peak was observed, followed by a small but significant PAP peak

which was eluted from the column with CBP2. The remaining PAP were eluted by the acetic acid/C1q buffer wash. When the experiment was repeated with LHRH at an equimolar concentration, there was no significant elution of PAP due to the LHRH wash (see FIG. 9B). These results show that PAP binding to the column was dependent on CBP2.

D. Controls

1. The low efficiency of PAP elution by the CBP2 solution could have been explained by either: 1) the presence of inactive CBP2 aggregates effectively lowering the free CBP2 concentration; or 2) because the PAP elution occurred under nonequilibrium conditions. Under nonequilibrium conditions, the CBP2 peptide would not adequately compete with the solid phase CBP2 for binding sites on the PAP since a high affinity interaction (i.e., a functional affinity) between the solid phase CBP2 and the bound PAP had been established.

To address the question of nonequilibrium conditions, a 1 ml sample of 20 μ g/ml PAP plus 200 μ g/ml CBP2 (0.1 mM; a ten times greater concentration than that required to give 50% inhibition of Ig-HRP binding to C1q) or 152 μ g/ml LHRH (0.1 mM) was incubated for 1 hour at 25° C. and then loaded on a pre-equilibrated CBP2 column and allowed to pass continuously over the column for 15 min. The sample was then eluted with 21 ml C1q buffer followed by 3 ml of 2% (v/v) acetic acid and then 6 ml C1q buffer. The column fractions were assayed for the presence of peroxidase as described above in Part A, and the concentration of PAP was calculated from the standard curve data.

The resulting elution profiles are presented in FIG. 10. The sample containing CBP2 and PAP exhibited a dramatic loss of PAP binding to solid phase CBP2 on the column, while the sample containing LHRH and PAP showed PAP binding to the column in the range normally observed. These data show that PAP binding was dependent on CBP2.

If inactive CBP2 aggregates caused the low efficiency of PAP elution, then the same aggregates would effectively reduce the CBP2 concentration and the inhibition of PAP binding in the pre-incubation studies would require very high CBP2 concentrations. This was not the case, however, since similar concentrations were used for column elution as for the pre-incubation studies. Also, another attempt at PAP inhibition in the pre-incubation studies using 156 μ g/ml CBP2 demonstrated inhibition of PAP binding (approximately 40%). Thus, the effect of aggregates, if they formed, was of little significance.

2. In order to address the possible effects of acetic acid exposure on PAP, a 20 μ g/ml sample of PAP was dissolved in either 2% (v/v) acetic acid or C1q buffer and incubated for 3 hours. Aliquots from both samples were then assayed for peroxidase activity. No significant differences in the two samples were observed. Therefore, an adverse effect of acetic acid on HRP did not account for the results.

3. Although the data indicated that PAP binding was specific and dependent on CBP2, the PAP binding could have been due to a nonspecific interaction of the column with the horseradish peroxidase (HRP) of PAP. Thus, a solution of HRP in C1q buffer was loaded, eluted and assayed for peroxidase as described for the PAP experiments. The linear regression calculations of the resulting profile indicated no significant binding of HRP (only about 7%) to the CBP2 column.

4. The extent of nonspecific binding of PAP to the HIPACTTM matrix was determined. Control columns coupled with either LHRH or No peptide were used in place of the CBP2, in order to assess the extent of nonspecific binding.

In the same manner as described for the CBP2 column, PAP were loaded and eluted either from the LHRH or No peptide column. The column fractions were assayed for the presence of immunoglobulin using the enzyme immunoassay described above in Part B. Linear regression analysis of the standard curves allowed the calculation of the amount of material in the flow-through and acid-eluted fractions.

The results are shown in Table 1 above. The LHRH column bound 4.8% of the total PAP loaded on the column, while the No-peptide column bound 8% of the total PAP. Therefore, the nonspecific binding of PAP to the CBP2 column can account for, at most, a small fraction of the total PAP binding observed.

E. Conclusion

The data show that the binding of PAP to the CBP2 column was specific for immune complexes and dependent on CBP2, and that CBP2 was interacting with the immunoglobulin components of the PAP complex.

Example 5

Aggregated Human IgG Binding to the CBP2 Column

Human IgG was aggregated with alkali according to the method of Jones et al., *J. Immunol. Meth.*, 53, 201-208 (1982). Alkali-aggregated IgG possesses C1q binding activities similar to those of native immune complexes, thereby satisfying the essential criterion for immune complex models.

A 1 ml sample of 100 µg/ml of the aggregated IgG diluted in C1q buffer was loaded onto a CBP2 column (prepared as described in Example 3) that had been equilibrated with 3 ml C1q buffer. The sample was circulated through the column for 15 min and then eluted with 21 ml C1q buffer, followed by 3 ml 2% (v/v) acetic acid, and then by 6 ml C1q buffer.

The fractions were assayed for immunoglobulin by placing duplicate 50 µl aliquots from each fraction and 50 µl of coating buffer into the wells of a microtiter plate and incubating the plate 2 hours at 37° C. The uncoated surfaces were blocked by adding 340 µl/well of PBSC followed by incubation of the plate for 1 hour at 37° C. Next, the wells were washed 3 times with PBSCT. Goat anti-human IgG (Heavy and Light chain specific) F(ab')₂ fragments labeled with horseradish peroxidase (Organon Teknica/Cappel, West Chester, Pa.) was added to the wells (100 µl/well) at a 1:30,000 dilution in PBSCT, and the plate was incubated for 2 hours at 25° C. The wells were washed 3 times with PBSCT. OPD substrate buffer added (100 µl/well), and the resultant colored product was measured at 414 nm with a Titertek Multiskan plate reader. A standard curve using a portion of the sample loaded on the column was used in the assay in order to quantitate the amount of IgG in each column fraction.

The elution profile for the aggregated IgG showed two major peaks (FIG. 11). The aggregates not bound by the column were readily eluted with C1q buffer and were found to make up the first, flow-through peak. The flow-through material was calculated to comprise 5% of the total aggregated IgG added to the column. The aggregates bound to the column were eluted with the acetic acid wash and constituted the larger second peak (FIG. 11). Linear regression calculations indicated that 95% of the aggregated IgG loaded on the column was bound to the column and eluted with the acetic acid wash. Based on these data, the efficiency of the CBP2 column for binding aggregated IgG was considered quite high.

Next, a 1 ml sample of 400 µg/ml monomeric human IgG was diluted in C1q buffer, loaded on a CBP2 column, eluted and assayed in the same fashion as described above for the aggregated human IgG. Also, a one ml sample containing a mixture of 100 µg/ml aggregated human IgG and 400 µg/ml monomeric human IgG in C1q buffer was loaded, eluted, and assayed as described above. The results are shown in FIG. 12 and Table 2 below.

TABLE 2

Sample	Percent of Added Material Bound to the Column		
	CBP2 Column	LHRH Column	No Peptide Column
Aggregated IgG	96.7	18.8	26.3
Monomeric IgG	31	N.D.*	N.D.*
Aggregated + Monomeric IgG	95.9 ^b	N.D.*	N.D.*

*Not Determined

^bThe value shown is the percentage of aggregated IgG bound. The aggregates were biotinylated allowing for a separate determination (see below).

The linear regression analysis calculations indicated that approximately 31% of the total monomeric IgG loaded on the column was bound (see Table 2). This amount of binding of the human monomeric IgG was quite high relative to previous binding experiments using monomeric rabbit IgG. However, there was a significant difference in the amount of binding of aggregated IgG and that of monomeric IgG.

The linear regression calculations for the mixture of aggregated and monomeric human IgG determined that there were 100 µg of IgG in the acid-eluted fractions, indicating that the column bound almost all of the aggregated IgG. Although the calculated amount of IgG in the acid-eluted fractions closely corresponded to the amount of aggregated IgG loaded onto the column, there was no evidence indicating whether or not the aggregated IgG was actually the species binding to the column. It was, therefore, necessary to be able to distinguish the aggregated IgG in a mixture of monomeric IgG and aggregates.

To do this, a 5 mg/ml preparation of aggregated human IgG was labeled with sulfosuccinimidyl-6-(biotin-amido) hexanoate (NHS-LC-Biotin, Pierce Chemical Co., Rockford, Ill.) according to the method of Geudson et al., *J. Histochem. Cytochem.*, 27, 1131-39 (1979). The aggregated IgG had 14% of the available primary amines biotinylated as determined by the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method described in Fields, *Meth. Enzymol.*, 25B, 464-68 (1972). Biotinylation of the aggregates using an N-hydroxysuccinamide ester adduct of biotin allowed the rapid and specific labeling of the aggregates. Biotinylation has been demonstrated to have minimal effects on the native conformations and activities of biomolecules, and CBP2+biotin showed no difference in its Protein A inhibitory activity. In addition, the avidin-biotin interaction is of a very high affinity (femtomolar in magnitude) providing a highly specific and extremely sensitive label for the aggregated IgG.

Next, a 1 ml sample containing biotinylated aggregated human IgG at 100 µg/ml plus monomeric IgG at 400 µg/ml in C1q buffer was loaded, eluted, and the column fractions assayed for immunoglobulin as described above. The fractions were also assayed for biotinylated aggregates as follows. Duplicate 50 µl aliquots of each column fraction were mixed with 50 µl coating buffer in the wells of a microtiter

plate, and the plate was incubated for 2 hours at 37° C. The uncoated surfaces of the wells were blocked with PBSC. Avidin plus biotinylated horseradish peroxidase (Vector Laboratories, Inc., Burlingame, Calif.) (5 µl of each) diluted in 1 ml PBSCT were incubated for 30 min at 25° C. and then diluted 1:15 in PBSCT prior to adding 100 µl/well of the resulting complexes to the plate. The plate was incubated for 2 hours at 25° C., the wells washed 3 times with PBSCT, and OPD substrate buffer added, and the colored product measured at 414 nm in a Titertek Multiskan plate reader.

The results are shown in FIGS. 13A (assay for immunoglobulin) and 13B (assay for biotinylated aggregated IgG) and Table 2 above. The concentration of biotinylated aggregated IgG was calculated from the standard curve and linear regression analysis, and it was found that 95.6% of the biotinylated aggregated IgG loaded on the column was bound by the column (see Table 2), which indicates that immune complexes can be bound to the CBP2 column specifically in the presence of monomeric immunoglobulin.

As a control, C1q was coated on the wells of a microtiter plate as described in Example 2, and aliquots from each column fraction were incubated with the solid phase C1q for 2 hours at 25° C. The wells were washed 3 times with C1q buffer. Bound immune complexes were assayed as described above using goat anti-human IgG F(ab')₂ fragments. Such antibody fragments cannot be bound by C1q, since the fragments have no Fc region.

The results of this assay of C1q binding activity showed that all of the C1q binding material was in the acid-eluted fractions, which are the fractions containing aggregated IgG that bound to the CBP2 column (see FIG. 13C). The combined data show that the CBP2 column can bind human immune complexes specifically, and that the CBP2 column binds immune complexes which are bound by C1q.

The binding of aggregated IgG by the control columns is also shown in Table 2. Approximately 19% of the total aggregates added to the LHRH column were bound to it, while approximately 26% of the total aggregates bound to the No-peptide column. Although the nonspecific binding of the aggregate to the matrix is 19-26%, the binding of aggregated IgG to the CBP2 column (95% of total aggregated IgG) clearly cannot be explained by nonspecific effects alone. Based on the relatively high percentage of nonspecific binding to the matrix, the monomeric IgG binding to the column may be attributable to nonspecific binding.

In conclusion, the above data show that the CBP2 column binds immune complexes that are also bound by C1q and that the CBP2 column is binding immune complexes in a specific fashion.

Example 6

Binding of Aggregated IgG and Serum Components to the CBP2 Column

Biotinylated aggregated IgG (100 µg/ml), prepared as described in Example 5, was diluted in normal human plasma which had been diluted 1:20 with C1q buffer. A 1 ml sample of the aggregated IgG in diluted human plasma was applied to a CBP2 column (prepared as described in Example 3) and eluted with C1q buffer, followed by a 2% (v/v) acetic acid wash to elute the bound material. The column fractions were assayed for immunoglobulin using goat anti-human IgG F(ab')₂, as described in Example 5, and the results are shown in FIG. 14A. The biotinylated aggregated IgG was detected with avidin-biotinylated-HRP com-

plexes as described in Example 5, and the results are shown in FIG. 14B. Finally, C1q binding activity was assessed as described in Example 5, and the results are shown in FIG. 14C.

Nearly all of the biotinylated aggregates bound to the column as can be seen in FIGS. 14B and C. The calculated total aggregates bound was 98.2% of the amount added as determined from the linear regression analysis of the accompanying standard curve. Thus, the efficiency of immune complex binding in dilute plasma was quite high.

In view of the success of this experiment, human serum samples were tested on the CBP2 column. The levels of immunoglobulin and immune complexes in the sera were determined prior to their passage over the CBP2 column to establish baseline readings. To do so, either Protein A or C1q was coated on the wells of a microtiter plate as described in Example 2, and then 1:100 diluted serum samples were incubated with the solid phase Protein A or C1q. Any bound material was detected using goat anti-human IgG F(ab')₂ fragments labeled with HRP as described in Example 5. A standard curve of aggregated IgG was also included with each assay and provided a way to normalize readings between the two assays (i.e., in aggregated IgG equivalents).

The calculated aggregated IgG equivalents for both the Protein A and C1q assays of the serum samples before passage over the column are presented in Table 3. Ratios of the amounts of material bound by C1q to the amounts of material bound by Protein A, and expressed as percentages, are presented in FIG. 15. The Protein A assay showed that the sera had concentrations of immunoglobulin (IgG) ranging from 726.9 to 1336.2 µg/ml, while the corresponding C1q assay showed immune complex (IC) levels ranged from 7.1 to 32.1 µg/ml (see Table 3).

TABLE 3

Amounts of IgG or Immune Complexes (IC) determined by assay (µg/ml)

Serum Number	Protein A (IgG)	C1q (IC)
325	725.9	32.1
332	749.1	17.6
318	1183.1	17.3
335	1336.2	7.1
321	1175.7	11.0

Next, the serum samples were diluted 1:20 with C1q buffer and loaded onto a CBP2 column. The column was washed with C1q buffer, and the bound material eluted from the column with 2% (v/v) acetic acid. The column fractions were assayed for immunoglobulin as described in Example 5. From the resulting elution profiles and the linear regression analysis of the standard curves, the amount of immunoglobulin passed over the column (total IgG) and the amount of material bound (acid-eluted fractions) were determined, and the results are presented in Table 4.

TABLE 4

Amounts of IgG (µg)

Serum Number	Total IgG		Acid-Eluted Fractions	
	Calculated ¹	Actual ²	Calculated ¹	Actual ²
325	36.3	126.8	1.61	4.73
332	37.5	49.4	0.88	2.87
318	59.2	33.3	0.87	1.72

TABLE 4-continued

Serum Number	Amounts of IgG (μ g)			
	Total IgG		Acid-Eluted Fractions	
	Calculated ¹	Actual ²	Calculated ¹	Actual ²
335	66.8	84.77	0.36	5.08
321	58.8	40.5	0.55	4.59

¹Calculated amount in serum before it was passed over the column using Protein A and C1q binding assays.

²Calculated in the present experiment using the assay for IgG.

To the acid-eluted fractions, 100 μ l of 3.4M NaOH was added to neutralize the acetic acid. The column fractions were then divided into two groups (the flow-through and the acid-eluted fractions), pooled and then dialyzed against PBS overnight at 4° C. The dialyzed fractions were then aliquoted and stored at -70° C. until the samples could be analyzed as described below.

First, a 1 μ g/ml solution of Protein A in coating buffer was incubated in the wells of a microtiter plate (100 μ l/well) for 2 hours at 25° C. The uncoated surfaces were blocked with PBSC as described in Example 2. Aliquots (100 μ l/well) of both the acid-eluted and flow-through fractions were incubated with the solid phase adsorbed Protein A for 2 hours at 25° C. The wells were then washed 3 times with PBSCT, and goat anti-human IgG F(ab')₂ labeled with horseradish peroxidase diluted 1:30,000 in PBSCT was added (100 μ l/well). The plate was incubated for 2 hours at 25° C. The wells were washed 3 times with PBSCT, followed by the addition of OPD substrate buffer. The colored product was measured at 414 nm using a Titertek Multiskan plate reader.

Also, C1q was coated onto the wells of a microtiter plate. A 10 μ g/ml solution of C1q in coating buffer was added to the wells of a microtiter plate (100 μ l/well), and the plate was incubated for 2 hours at 25° C. The wells were blocked with PBSC as described in Example 2. Samples (100 μ l/well) from both the acid-eluted fraction and the flow-through fraction were incubated with the solid phase C1q for 2 hours at 25° C. The wells were washed 3 times with PBSCT, and goat anti-human IgG F(ab')₂ was added at a 1:30,000 dilution in PBSCT (100 μ l/well), and the plate was incubated for 2 hours at 25° C. PBSCT was used to wash the wells 3 times, OPD substrate buffer added, and the colored product measured at 414 nm in a Titertek Multiskan plate reader.

A standard curve of aggregated human IgG was used for both the C1q and protein A binding assays. The use of a common standard curve facilitated a comparison of the results from the two assays, and results were expressed in aggregated IgG equivalents.

The amounts of immunoglobulin (Protein A binding material) and immune complexes (C1q binding material), expressed in aggregated IgG equivalents are presented in Table 5. Also, the amount of immune complexes as a percentage of total immunoglobulin is presented in FIG. 16.

TABLE 5

Serum Number	Amounts of IgG or Immune Complexes (IC) in Column Fractions (μ g/ml)			
	Flow-Through		Acid-Eluted	
	Protein A (IgG)	C1q (IC)	Protein A (IgG)	C1q (IC)
325	39.6	0.10	0.30	0.074
332	25.6	0.082	0.16	0.095
318	123.1	0.11	0.16	0.097
335	19.7	0.27	0.14	0.13
321	15.7	0.23	0.161	0.159

The C1q binding activity detected in the flow-through fractions was a small percentage of the total IgG as determined by Protein A binding (Table 5). The acid-eluted fractions had a much higher percentage of C1q binding material when compared to the Protein A reactive material (Table 5). In some cases it was as high as 98%. By comparing the results presented in FIG. 16 with the results in FIG. 15, it can be seen that the acid-eluted fractions were enriched with immune complexes (as detected by the C1q assay).

The efficiency of the column [the ratio of the total amount of C1q bound material in the pooled acid-eluted fractions versus the amount of C1q binding material in a 1 ml sample of 1:20 diluted serum before passage over the column] ranged widely from 23% to 109%. This variability may be due to the fact that sera vary in their immune complex content due to the variation from person to person. The CBP2 column and the C1q assay may also be detecting different species of immune complexes. C1q has very distinct binding affinities for IgG subclasses and for IgM, but the immunoglobulin specificity of the CBP2 column remains to be determined.

Example 7

HiPAC™ Fast Protein Liquid Chromatography (FPLC) Column

A. CBP2 Coupling to HiPAC™ FPLC Column.

CBP2 was coupled to an HiPAC™ FPLC column (dimensions of 0.6 mm \times 10 cm) in the following fashion. The column was equilibrated with 10 column volumes (18 ml) of 0.1M sodium citrate, pH 5.5, at a flow rate of 1 ml/min at 25° C. A ligand solution of 1.4 mg/ml CBP2 diluted in 0.1M sodium citrate, pH 5.5, plus 20 mg/ml sodium cyanoborohydride was prepared, and a 3 ml sample applied to the column. The sample was circulated through the column for 20 min at 25° C. at a flow rate of 1 ml/min using a peristaltic pump (Rainin Inc., Woburn, Mass.). The column was then washed with 20 ml of sodium citrate, pH 5.5, at 1 ml/min followed by 10 ml of 2M guanidine-HCl, and then 10 ml of 0.05M Tris, pH 9.0, at the same flow rate. The column was then washed with 20 ml C1q buffer and stored in C1q buffer for future use. This column had a bed volume of 1.8 ml as compared to 0.8 ml for the LTQ column.

The extent of CBP2 coupling to the column was determined by directly assaying the column with Ellman's reagent as described in Example 3. The amount of CBP2 coupled to the column as determined by this assay was 756 μ g. The density of CBP2 in relation to the column volume was 420 μ g CBP2/ml matrix. This is 20% less than the density obtained for the LTQ column, which was 525 μ g CBP2/ml matrix.

B. Aggregated Human IgG Binding to the FPLC-CBP2 Column.

The CBP2/FPLC column was equilibrated with C1q buffer at a flow rate of 1 ml/min for 15 min using a Beckman HPLC (Model 421 controller and 112 solvent delivery module, Palo Alto, Calif.). A 2 ml sample of 100 µg/ml aggregated human IgG (prepared as described in Example 5) diluted in C1q buffer was loaded onto the column with C1q buffer at a flow rate of 1 ml/min for 2 min. The flow rate was then increased to 2 ml/min, and maintained for 20 minutes, at which time the flow rate was reduced to 1 ml/min. The column was next washed with 2% (v/v) acetic acid at a flow rate of 1 ml/min for 12 min, followed by C1q buffer at a flow rate of 1 ml/min for 6 min. The column eluate was collected at 1 minute intervals and assayed for the presence of immunoglobulin as described in Example 5.

The resulting profile is presented in FIG. 17. From the linear regression calculations, the concentration of aggregated IgG in each column fraction was determined. According to the calculations, 96% of the total aggregated IgG added to the column was found in the acid-eluted fractions. Therefore the column was able to bind immune complexes with a high efficiency.

In order to determine the specificity of the FPLC-CBP2 column for immune complexes, monomeric human IgG was loaded and eluted from the column and assayed as described above for the aggregated IgG. The linear regression calculations determined that 13% of the monomeric IgG loaded on the column was bound to the column, indicating that the FPLC-CBP2 column was in fact specific for immune complexes and more specific than the LTQ column which exhibited 31% binding of monomeric human IgG.

Next, biotinylated aggregated IgG at 100 µg/ml was added to human plasma diluted 1:20 in C1q buffer, and a 2 ml aliquot of the sample was loaded and eluted from the CBP2-FPLC column as described above and then assayed for total IgG and biotinylated aggregated IgG as described in Example 6.

The resulting profiles are presented in FIG. 18. FIG. 18 shows extensive trailing of the flow-through material, with a drop in IgG concentration prior to the acid-elution fractions. The acid-elution fractions have a peak of IgG. The aggregated IgG profile in FIG. 18 shows that the aggregates remained bound to the column despite extensive flow-through of excess IgG, until they were eluted by the acid wash. Based on linear regression analysis of the aggregated IgG profile, nearly all of the biotinylated aggregated IgG was bound by the column. The data also indicated that the FPLC-CBP2 column could bind immune complexes preferentially in the presence of free monomeric IgG, in a fashion similar to the LTQ-CBP2 column.

C. Human Serum Components Binding to the FPLC-CBP2 Column.

Sera were diluted 1:20 in C1q buffer and loaded and eluted as described above, and the column fractions assayed as described in Example 6. The results are presented in Tables 7 and 8 and FIG. 19.

Table 7 shows the total IgG in the initial serum sample ("calculated"), the total IgG found in the eluted fractions after the serum samples were passed over the column ("actual"), the amount of immune complexes bound to the column and eluted with the acid wash (acid-eluted fractions) ("actual"), and the amount of immune complexes in the

initial sample ("calculated"). FIG. 19 shows the ratio of C1q binding material to Protein A binding material for each serum sample based on the values in Table 8 which shows the amounts of Protein A and C1q binding material in the pooled flow-through and acid-eluted fractions.

TABLE 7

Serum Number	Total IgG (µg)		Acid-Eluted Fractions (µg)	
	Calculated ¹	Actual ²	Calculated ¹	Actual ²
325	72.6	254.5	1.76	0.376
332	75.0	130.8	1.76	3.86
318	118.2	138.62	1.74	2.33
335	133.6	141.64	0.72	1.71
321	117.6	280.79	1.10	3.02

¹Calculated amount in serum before passage over the column as determined by binding to Protein A or C1q.

²Calculated amount in fractions after passage over the column; calculated using assay for IgG.

TABLE 8

Amounts of IgG or IC in Column Fractions (µg/ml)

Serum Number	Flow-Through		Acid-Eluted	
	Protein A Binding (IgG)	C1q Binding (IC)	Protein A Binding (IgG)	C1q Binding (IC)
325	83.2	0.0795	0.188	0.0458
332	42.7	0.342	0.68	0.0291
318	73.2	0.0867	0.497	0.224
335	27.0	2.45	0.270	0.116
321	82.9	1.45	0.584	0.178

Based on the data, the FPLC-CBP2 column appeared to bind immune complexes in a similar fashion as the LTQ-CBP2 column. The FPLC-CBP2 column, like the LTQ column, also demonstrated a significant enrichment for C1q binding material in the acid-eluted fractions.

The FPLC-CBP2 column efficiencies differed significantly from the LTQ column efficiencies with regards to sera 325 and 332 (Table 9). Both columns demonstrated high binding efficiencies for sera 335 and 321, while serum 318 was bound with a moderate efficiency by both columns.

TABLE 9

Serum Number	Efficiency	
	CBP2-LTQ Column	CBP2-FPLC Column
325	23%	8.54%
332	54%	9.94%
318	56%	78.1%
335	109%	96.7%
321	87%	97.2%

*The efficiency of binding was calculated as the percentage of actual ICs in the acid-eluted fractions over the calculated ICs in the sample added to the column.

In conclusion, based on similar trends in immune complex binding efficiencies and enrichments, the FPLC-CBP2 column was determined to be a reasonable scaled-up version of the LTQ-CBP2 column.

33

34

Leu Glu Gln Gly Glu Asn Val Phe Leu Gln
1 5 10

[SEQ ID NO 3]

6. The device of claim 4 wherein the binding material is
a solid phase having the binding peptides attached thereto.

Ala Thr Leu Leu Cys.
15

5

* * * * *

DOCUMENT-IDENTIFIER: US 5268363 A

TITLE: Method of treatment to inhibit the undesirable activation of the complement cascade with factor J, an inhibitor of complement C1

BSPR:

The activation of the complement cascade can also cause undesirable phenomena, such as inflammation, damage of normal tissue and disease states such as the autoimmune diseases. Autoimmune diseases are associated with the immune

complexes formed against indigenous tissue which are associated with the biologically active complement fragments generated by the classical portion of the complement cascade. Such diseases include but are not limited to:

Hashimoto's thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, Graves' disease, myasthenia gravis, insulin resistance, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, and rheumatoid arthritis.

BSPR:

In terms of the regulation of the complement system, most studies have focused on the binding properties of the C1 serine proteinase subcomponents, C1r and C1s, for a serum glycoprotein, C1 Inhibitor. Another Inhibitor that has been identified but whose role in regulating C1 function in plasma is not clear is the C1q inhibitor (C1qINH).

BSPR:

This inhibitor is functionally and antigenically distinct from other known inhibitors of C1, namely, C1INH and C1qINH. The inhibitory capabilities of C1 Inhibitor are the result of its binding to the catalytic subunits of C1, C1r and C1s, and thereby inhibiting C1r and C1s. The C1q Inhibitor can only inhibit the assembly of the C1 complex by prior binding to C1q. In contradistinction, it has been discovered that factor J does not

inhibit C1s,
and that factor J can both dissociate intact C1 as well as
prevent its assembly
from subcomponents. Thus, factor J is functionally distinct from
C1 Inhibitor
and Clq Inhibitor.

DEPR:

Factor J did inhibit association of the C1 complex as measured by
factor J's
ability to inhibit the precipitation of .sup.125 I-Clq in the
presence of C1r
and C1s, FIG. 7. This titration profile, 71, was very similar to
that obtained
when the dose response of factor J inhibition of C1 formation in
the hemolytic
assay, 72. Both assays were measured over the same concentration
range of
polypeptide. The difference in the shape of the inhibition
curves for .sup.125
I-Clq interaction with C1r.sub.2 s.sub.2, 71, and the inhibition
of C1
hemolytic activity, 72, emphasizes that factor J inhibits the Clq
and C1r.sub.2
s.sub.2 reaction in a saturable manner consistent with direct
binding to C1,
whereas the inhibition of C1 hemolytic function follows a
sigmoidal 72 response
consistent with the complex kinetics of erythrocyte lysis induced
by diluted
serum.

DEPR:

Although factor J has been shown to inhibit Clq association with
C1r.sub.2
s.sub.2, the mechanisms for this inhibition are not the same as
that of C1
inhibitor or Clq inhibitor. C1 inhibitor acts by binding to both
catalytic
subunits of C1, C1r and C1s. An assay measuring esterase
activity of purified
C1s, FIG. 8 compares factor J inhibition for C1s, 82, with
inhibition of the C1
inhibitor for C1s, 83. As seen in FIG. 8, C1s in the presence of
factor J, 82,
or buffer alone, 81, show comparable amounts of esterase
activity, whereas, the
addition of C1 Inhibitor resulted in a significant decrease in C1
esterase
activity, 83. Clq inhibitor acts by binding to Clq and thereby
preventing the

catalytic subunits from binding to Clq. FIG. 9 demonstrates that factor J does

~~not bind to Clq under conditions in which the Clq Inhibitor could~~
bind Clq.

Partially purified Clq inhibitor bound to and precipitated

.sup.125 I-Clq, 101,

whereas, purified factor J did not bind to .sup.125 I-Clq in the fluid phase to

permit precipitation of the .sup.125 I-Clq, 102.

ORPL:

Silvestri et al., "The Clq Inhibitor in Serum is a Chondroitin 4-Sulfate

Proteoglycan" 256 J. Biol. Chem. 7383-7387 (1981).

ORPL:

Ghebrehiwet, "Clq Inhibitor (ClqINH): Functional Properties and Possible

Relationship to a Lymphocyte Membrane-Associated Cq1 Precipitin" 126 J.

Immunol. 1837-1842 (1981).

ORPL:

Ghebrehiwet, "Purification and Partial Characterization of a Clq Inhibitor from

the Membranes of Human Peripheral Blood Lymphocytes" 129 J.

Immunol. 151-162

(1982).



US005268363A

United States Patent [19]

Nicholson-Weller

[11] **Patent Number:** 5,268,363[45] **Date of Patent:** Dec. 7, 1993

[54] **METHOD OF TREATMENT TO INHIBIT THE UNDESIRABLE ACTIVATION OF THE COMPLEMENT CASCADE WITH FACTOR J, AN INHIBITOR OF COMPLEMENT C1**

[75] **Inventor:** Anne Nicholson-Weller, Wellesley, Mass.

[73] **Assignee:** The Beth Israel Hospital Association, Boston, Mass.

[21] **Appl. No.:** 874,620

[22] **Filed:** Apr. 27, 1992

Related U.S. Application Data

[62] Division of Ser. No. 406,144, Sep. 12, 1989, Pat. No. 5,109,114.

[51] **Int. Cl.⁵** C07K 3/28; A61K 37/02

[52] **U.S. Cl.** 514/21; 530/350; 530/412; 530/413; 530/415; 530/416

[58] **Field of Search** 514/21; 530/416, 412, 530/413, 415, 350

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,147,801	4/1979	Lenhard et al.	424/315
4,368,232	6/1983	Eibi et al.	530/380
4,883,784	11/1989	Kaneko	514/424
4,915,945	4/1990	Pelzer et al.	421/101
5,109,114	4/1992	Nicholson-Weller	530/350

OTHER PUBLICATIONS

Sofer et al. Nov./Dec. 1983, Bio/Techniques, pp. 198-203.

Cooper, "The Classical Complement Pathway: Activation and Regulation of the First Complement Component" 37 Adv. Immunol 151-216 (1985).

Schumaker et al., "Activation of the First Component of Complement" 5 Ann. Rev. Immunol. 21-42 (1987).

Reid et al., "The Proteologic Activation Systems of Complement" 50 Ann. Rev. Biochem. 433-464 (1981).

Ziccardi, "The Role of Immune Complexes in the Acti-

vation of the First Component of Human Complement" 132 J. Immunol. 282-288 (1984).

Ziccardi, "Demonstration of the Interaction of Native C1 with Monomeric Immunoglobulins and C1 Inhibitor" 134 J. Immunol. 2559-2563 (1985).

Silvestri et al., "The C1q Inhibitor in Serum is a Chondroitin 4-Sulfate Proteoglycan" 256 J. Biol. Chem. 7383-7387 (1981).

Ghebrehwet, "C1q Inhibitor (C1qINH): Functional Properties and Possible Relationship to a Lymphocyte Membrane-Associated Cq1 Precipitin" 126 J. Immunol. 1837-1842 (1981).

Ghebrehwet, "Purification and Partial Characterization of a C1q Inhibitor from the Membranes of Human Peripheral Blood Lymphocytes" 129 J. Immunol. 151-162 (1982).

Davis, "C1 Inhibitor and Hereditary Angioneurotic Edema" 6 Ann. Rev. Immunol. 595-628 (1988).

Lopez-Trascasa et al., 1989, J. Biol. Chem. 264(27): 16714-16221.

Abstract, Biosis No. 2064952 of Cheung et al. 1986, Biochem Biophys Acta 882(2): 200-209.

Ghebrehwet et al., 1988, J. Immunol Methods 110: 251-260.

Primary Examiner—Robert A. Wax

Assistant Examiner—Keith C. Furman

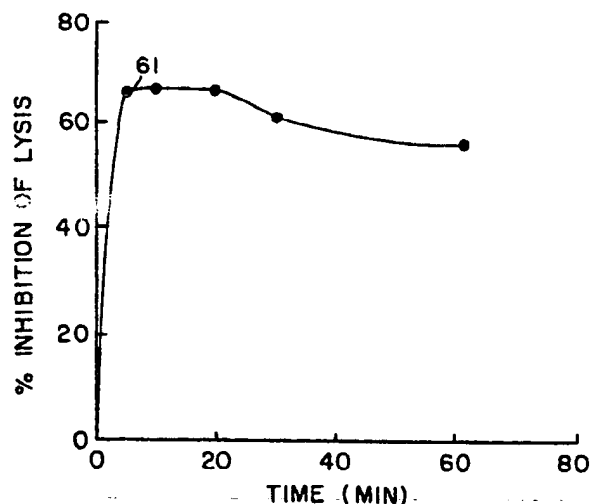
Attorney, Agent, or Firm—Lorusso & Loud

[57]

ABSTRACT

A purified protein, factor J, which has inhibitory properties which prevent the formation or the dissociation of C1 complex and a method of purification for said protein. The method including the following sequential chromatography steps: anion exchange QAE-"SEPHADEX", heparin-"SEPHAROSE" affinity, "MONO Q" and hydroxylapatite.

3 Claims, 6 Drawing Sheets



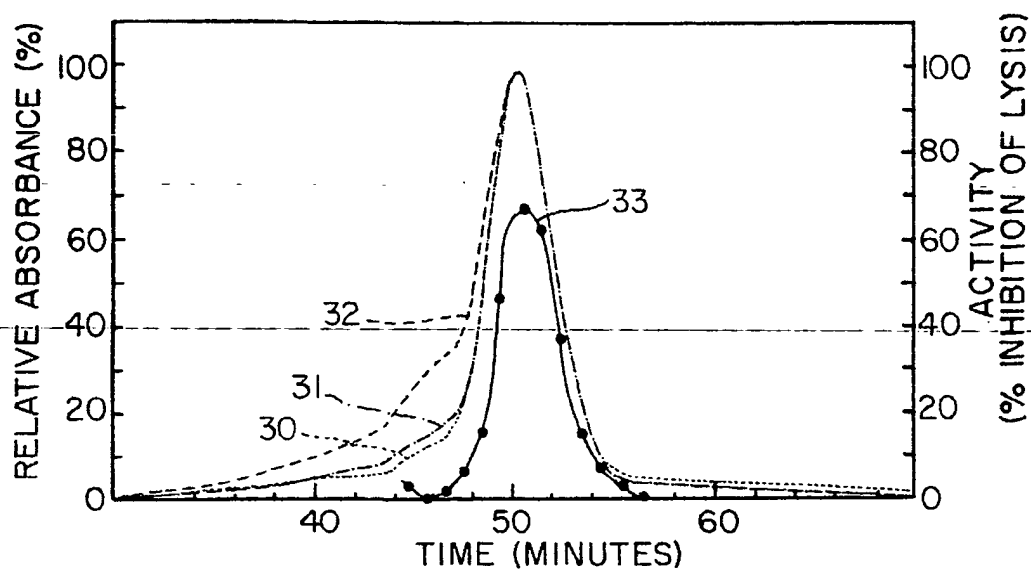


FIG. 1

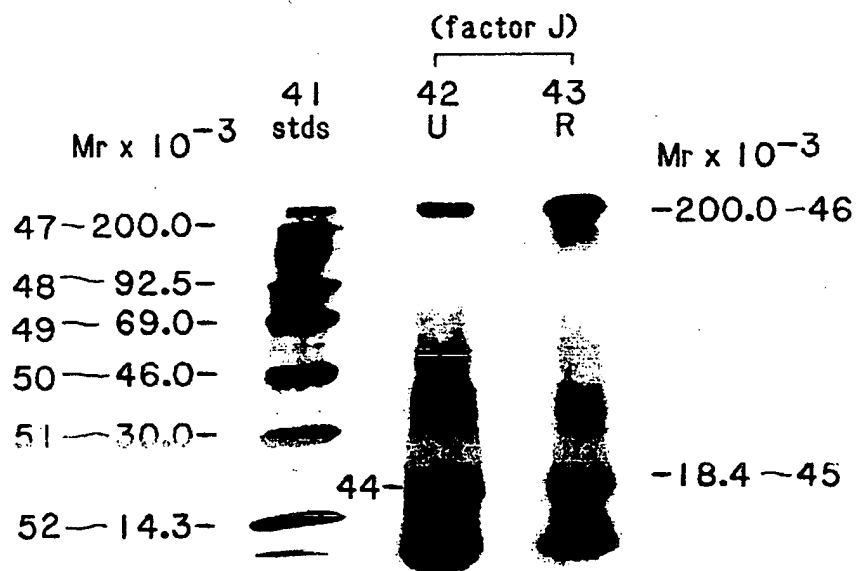


FIG. 2

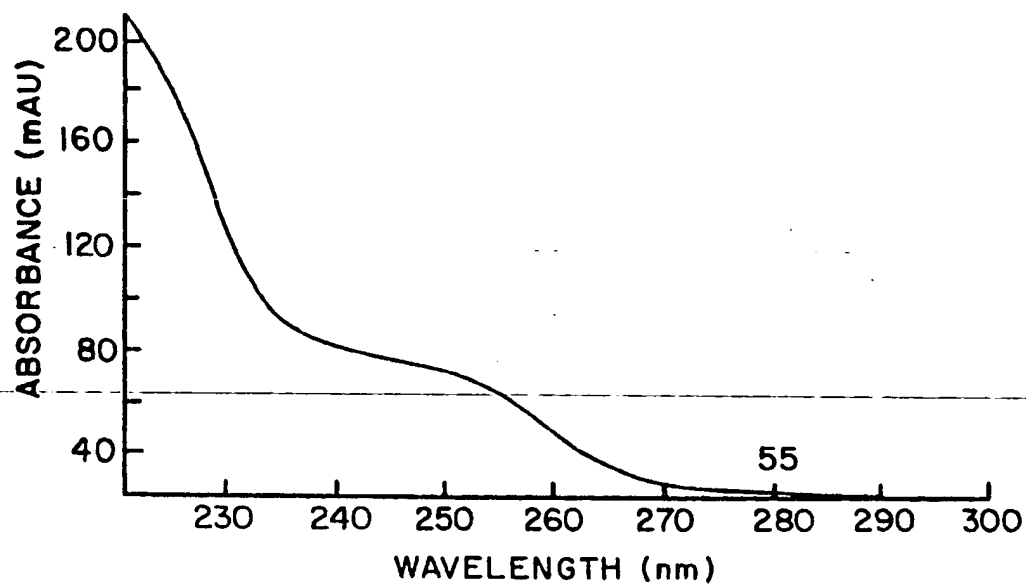
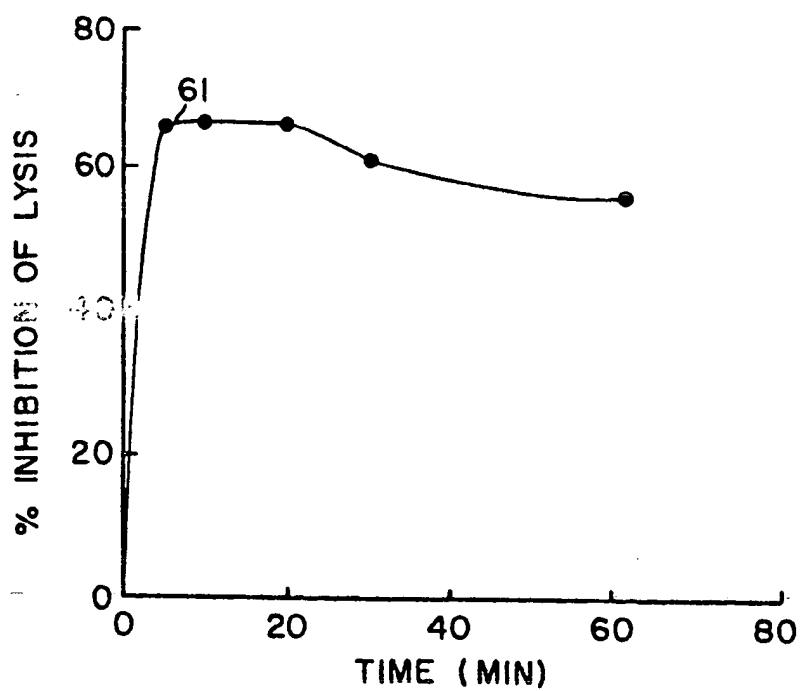


FIG. 3

FIG. 4



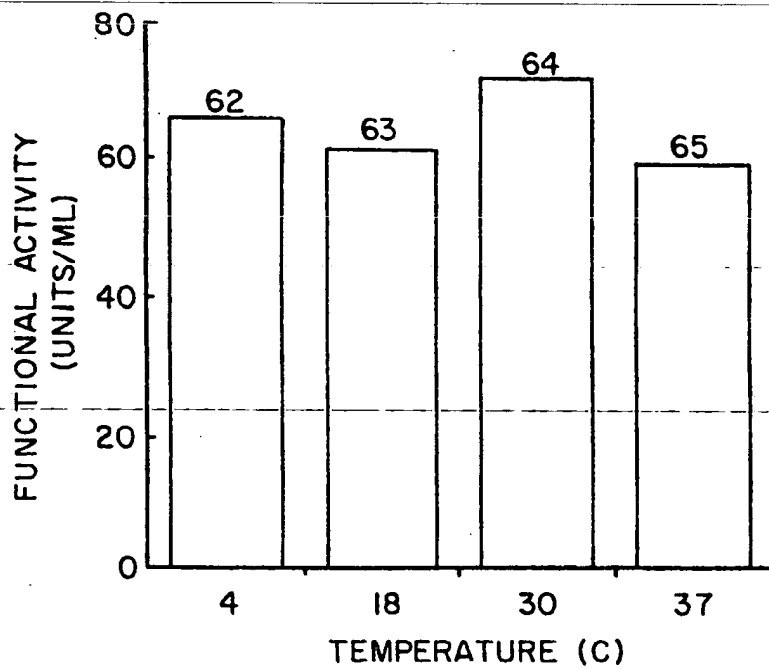
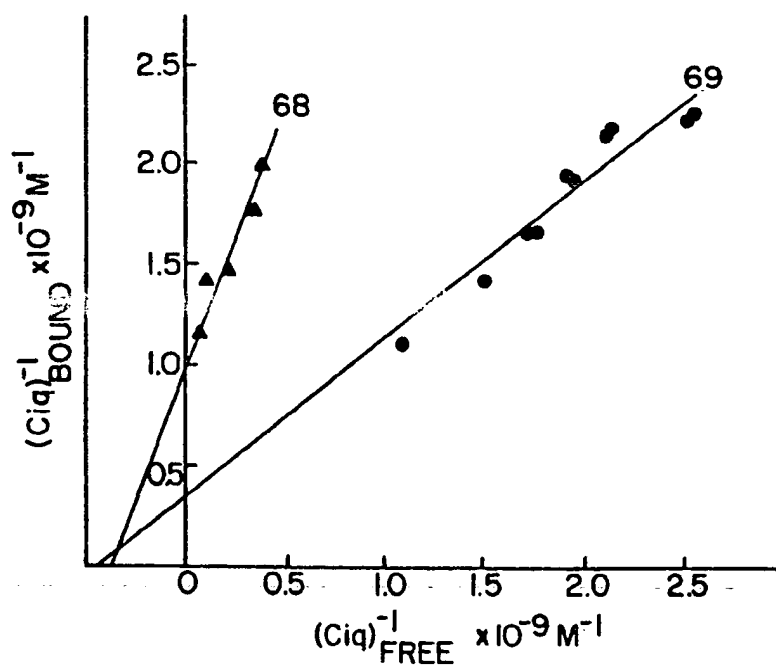


FIG. 5

FIG. 6



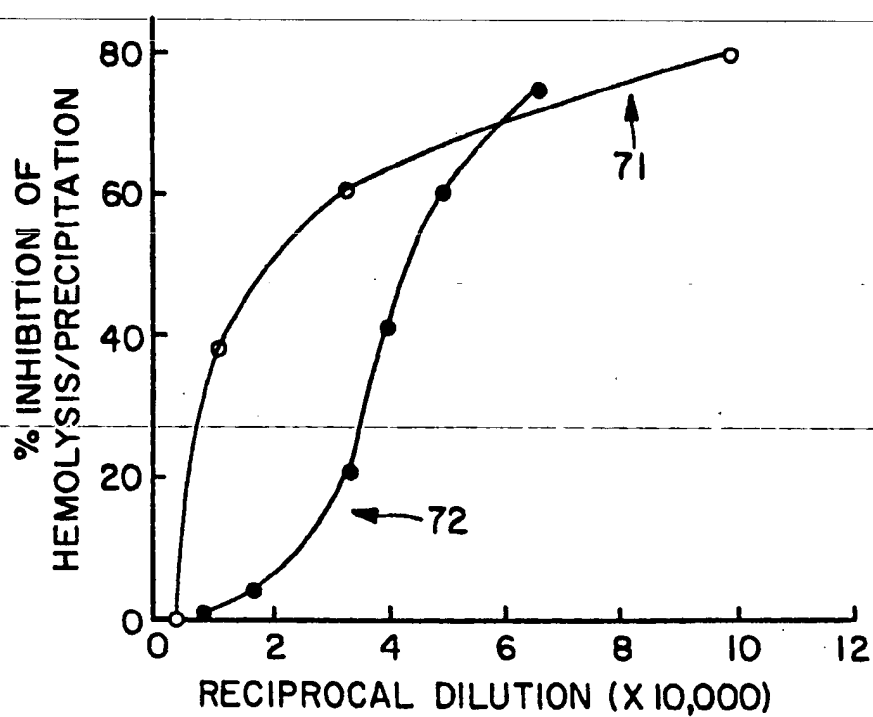


FIG. 7

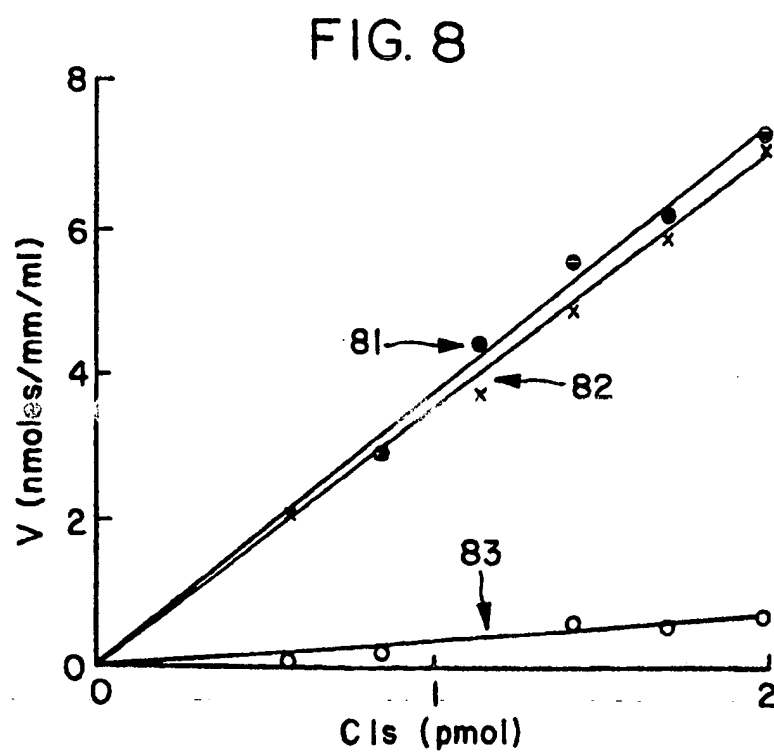


FIG. 8

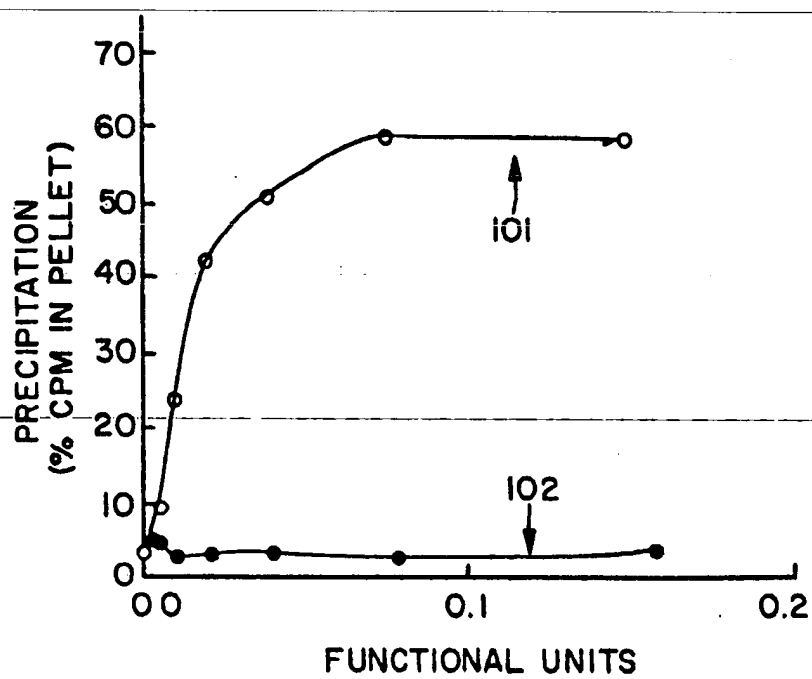


FIG. 9

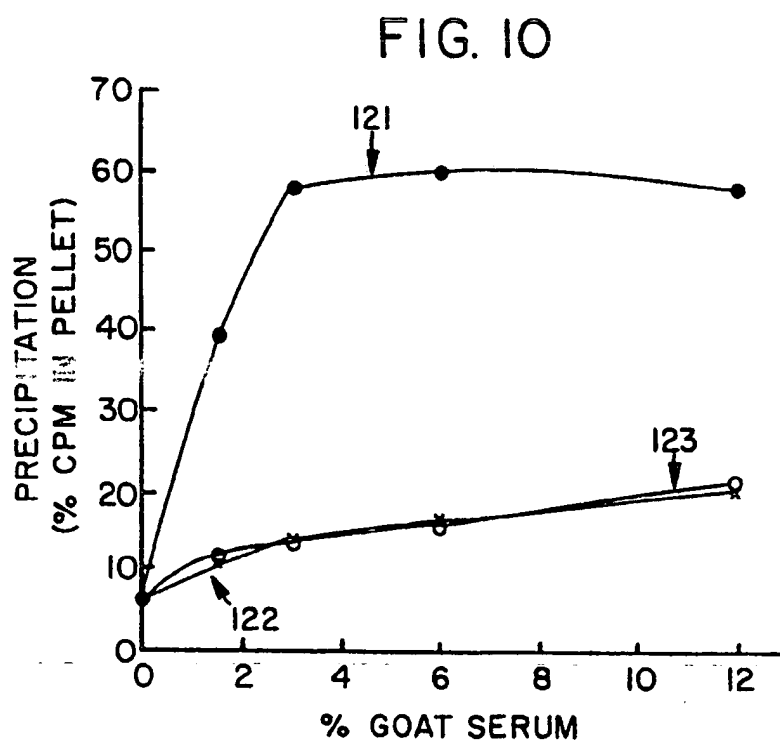
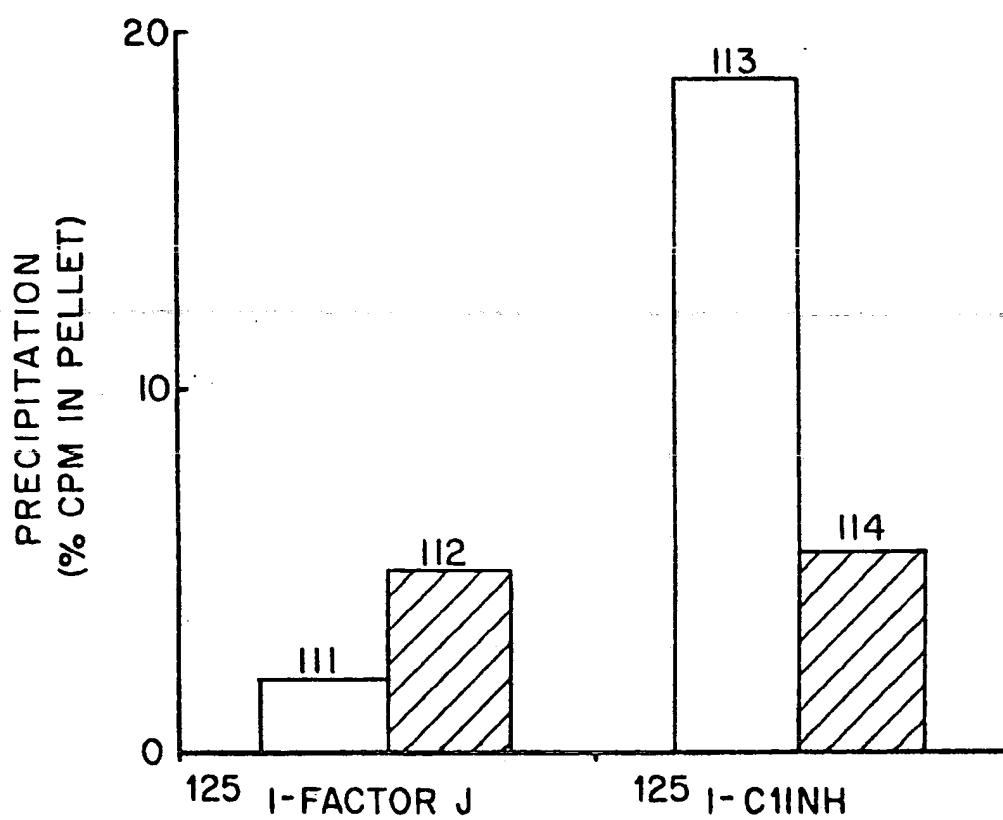


FIG. II



METHOD OF TREATMENT TO INHIBIT THE UNDESIRABLE ACTIVATION OF THE COMPLEMENT CASCADE WITH FACTOR J, AN INHIBITOR OF COMPLEMENT C1

BACKGROUND OF THE INVENTION

The invention described herein was made with Government support and the U.S. Government has certain rights in the invention.

"This is a divisional of copending applications(s) Ser. No. 07/406,144 filed on Sep. 12, 1989, now Pat. No. 5,109,114, which designated the U.S."

The immune system is the power of the body to resist invasion by pathogenic organisms, and to overcome such invasion and its ensuing infection, once it has taken place. The complement system is important in the immune response. Complement is a physiological process which involves many plasma proteins that react in a cascading (sequential) effect to mediate a number of desirable biologically significant phenomena. Such phenomena include modulation of the immune response, facilitation of the transport of immune complexes, production of anaphylatoxins which cause release of histamine, chemotaxis which is the migration of cells towards the area of complement activity, phagocytosis, and lysis of cells.

The activation of the complement cascade can also cause undesirable phenomena, such as inflammation, damage of normal tissue and disease states such as the autoimmune diseases. Autoimmune diseases are associated with the immune complexes formed against indigenous tissue which are associated with the biologically active complement fragments generated by the classical portion of the complement cascade. Such diseases include but are not limited to: Hashimoto's thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, Graves' disease, myasthenia gravis, insulin resistance, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, and rheumatoid arthritis.

It is known that the first phase of complement activation begins with C1. C1 is made up of three distinct proteins: a recognition subunit, C1q, and the serine proteinase subcomponents, C1r and C1s which are bound together in a calcium-dependent tetrameric complex, C1r₂s₂. An intact C1 complex is necessary for physiological activation of C1 to result. Activation occurs when the intact C1 complex binds to immunoglobulin complexed with antigen. This binding activates C1s which would then react with the next plasma protein, C4, to start the cascading effect rolling.

In terms of the regulation of the complement system, most studies have focused on the binding properties of the C1 serine proteinase subcomponents, C1r and C1s, for a serum glycoprotein, C1 inhibitor. Another inhibitor that has been identified but whose role in regulating C1 function in plasma is not clear is the C1q inhibitor (C1qINH).

It is important to identify and isolate inhibitors of the complement system because by isolating an inhibitor one may be able to control the effects of diseases such as those stated above. The inhibitors may provide a basis for pharmacologic intervention, either by allowing manipulation of the level of an inhibitor, or by providing a model for the chemical synthesis of a new inhibitor.

It is therefore an object of the present invention to provide a method for the isolation of an inhibitor of C1

which is functionally and antigenically distinct from known inhibitors of C1.

It is more specifically an object of the present invention to characterize the properties of an inhibitor of C1, factor J.

SUMMARY OF THE INVENTION

In accordance with the present invention, factor J is isolated from body fluid, in a multi-column purification procedure. The sequence of columns necessary for purification is an anion exchange, QAE "SEPHADEX" affinity, "MONO Q" and hydroxylapatite HPLC columns. The purified Factor J has a molecular weight (Mr) of about 20,000 daltons, minimal absorption at 280 nm, and a relatively small number of tyrosine residues. The newly discovered protein has been found to inhibit the association of the tetrameric complex C1r₂s₂ with the recognition subunit C1q, and it can dissociate the fully assembled-activated C1 complex.

This inhibitor is functionally and antigenically distinct from other known inhibitors of C1, namely, C1INH and C1qINH. The inhibitory capabilities of C1 Inhibitor are the result of its binding to the catalytic subunits of C1, C1r and C1s, and thereby inhibiting C1r and C1s. The C1q Inhibitor can only inhibit the assembly of the C1 complex by prior binding to C1q. In contradistinction, it has been discovered that factor J does not inhibit C1s, and that factor J can both dissociate intact C1 as well as prevent its assembly from subcomponents. Thus, factor J is functionally distinct from C1 Inhibitor and C1q Inhibitor.

In its broadest overall aspect, factor J is first isolated and purified and then administered in a therapeutic amount to inhibit the undesirable activation of the complement cascade.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an elution profile of the purified factor J of the present invention, at three different simultaneously recorded wavelengths (A 220, 254, and 280 nm).

FIG. 2 is an autoradiograph of radiolabeled factor J of the present invention, after being run on a SDS-PAGE 3-20% slab gel.

FIG. 3 is the UV spectra from 220-300 nm of the peak of factor J of the present invention, eluted from the hydroxylapatite column.

FIG. 4 is a graph showing the kinetics of factor J activity of the present invention as plotted on the y axis against the incubation time.

FIG. 5 is a bar graph which plots the temperature at which a hemolytic assay was performed on the x axis and the activity of factor J of the present invention (% inhibition of lysis using a hemolytic assay) on the y axis.

FIG. 6 is a reciprocal plot showing factor J of the present invention is a noncompetitive inhibitor.

FIG. 7 is a graph plotting the dose response of factor J of the present invention when titrated, x axis, in the hemolytic and C1 complex formation assays.

FIG. 8 is a graph showing the inability of factor J of the present invention to bind to the catalytic subunit of C1 as compared to C1 Inhibitor.

FIG. 9 is a graph of the addition of comparable amounts of functional activity of C1qINH or factor J of the present invention (x axis) plotted against the amount of bound complex ¹²⁵I C1 that is precipitable.

FIG. 10 is a graph indicating the presence of the factor J antigen of the present invention in human serum.

FIG. 11 is a bar graph showing the antigenic difference between C1 Inhibitor and factor J of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery of a new, functionally and antigenically distinct inhibitor of C1 complex association, factor J. A method is described for purifying and characterizing factor J.

The preparation starts with a sample of body fluid, such as, but not limited to, urine and serum, which has been dialyzed. The dialyzate is filtered and loaded onto an anion exchange column which has been equilibrated with dialysis buffer containing salt. The drop through fractions are collected and pooled.

The pooled fraction is diluted with the starting buffer quaternary-aminoethyl (QAE) "SEPHADEX" A-50 column and loaded onto the column. "SEPHADEX" is composed of beaded cross-linked dextran. Factor J is collected in the drop through and early eluted fractions of the linear salt onto a heparin "SEPHAROSE" affinity column. "SEPHAROSE" is composed of beaded agarose. Factor J elutes between 18 and 20 mS during a linear salt gradient when the column is equilibrated at pH 7.4 and NaCl provides the counter ion. Pools of fractions with factor J are concentrated and the buffer exchanged for the starting buffer of the "MONO Q" column with inhibitors. A MONO Q column is an anion exchanger having quaternary amine groups on polymeric resin beads having a pore structure of greater than 500 Angstroms used for the resolution of proteins and peptides.

The concentrated solution is loaded on the "MONO Q" column and the drop through fractions are pooled, concentrated and the buffer exchanged with phosphate starting buffer of the hydroxylapatite column. The solution is then loaded onto a hydroxylapatite column and eluted with an increasing linear phosphate gradient. Absorbances at 220, 250 and 280 are measured and the final pools are made based on the UV absorbency and inhibitory activity.

The following example is submitted to illustrate but not limit this invention.

EXAMPLE 1

Human urine was collected from normal donors in 250 ml polypropylene bottles containing stock amounts of the following inhibitors calculated to achieve the following final concentrations: 1 mM phenylmethylsulfonyl fluoride (PMSF); 5 mM EDTA; 0.01% sodium azide (NaN₃); 1 µg/ml leupeptin; 2 mM benzamidine-HCl, 1 µg/ml aprotinin. Upon collection of 250 ml of urine the bottle was frozen immediately at -70° C. To initiate the purification procedure the requisite number of bottles to provide 800-1000 ml urine were thawed, the urine adjusted to pH 7.4 with a saturated solution of Na₂HPO₄, and dialyzed in 3,500 M, cut-off tubing against 4 changes of 10 liters of 10 mM sodium phosphate, pH 7.4, 2 mM EDTA, 0.01% NaN₃, 0.5 mM PMSF buffer until the conductivity was 2 mS, or less. The dialyzed urine was filtered through a 3 µm pore polypropylene filter, available from Pall-Chisholm Company of Cranston, RI, and loaded onto a diethyl-amino ethyl (DEAE) "SEPHACEL" column, or DEAE "SEPHACEL" column, available from Pharmacia LKB Biotechnology of Piscataway, NJ, column (5×50 cm) equilibrated in the dialysis buffer with 0.04

M NaCl added. "SEPHACEL" is composed of beads of epichlorhydrin cross-linked cellulose. The drop-through fraction were pooled and the pool diluted with 5 volumes of 5 mM Tris buffer, pH 9, and applied to a QAE-"SEPHADEX" A-50, available from Pharmacia LKB Biotechnology of Piscataway, NJ., column (5×30 cm) equilibrated in 1 mM NaCl, 5 mM Tris, pH 9. Factor J activity was about equally present in the drop-through fractions and in the early eluted fractions when a linear gradient was applied of starting buffer made with 500 mM NaCl.

Separate pools of factor J were made from the drop-through and eluted fractions, and these pools were kept separate over the subsequent purification steps, although subsequent studies indicated there was no detectable difference in the factor J from the two pools. Each pool was loaded on a heparin-"SEPHAROSE" column (5×15 cm) made from crude porcine heparin coupled by cyanogen bromide to "SEPHAROSE" 4B available from Pharmacia LKB Biotechnology of Piscataway, NJ, and equilibrated in 25 mM NaCl, 50 mM Tris, pH 7.4. Factor J activity eluted between 18-20 mS during a linear gradient of starting buffer made with 1.5 M NaCl. Pools of fractions with factor J activity were concentrated and buffer exchanged by ultrafiltration using a cellulose 1000 M, cut-off membrane, Spectra/Por type C, available from Spectrum Medical Industries of Los Angeles, CA, into the starting buffer for the "MONO Q", 40 mM NaCl, 10 mM sodium phosphate, pH 7.8, 2 mM EDTA, 0.01% NaN₃, 5 mM PMSF. The concentrated pools were loaded onto a "MONO Q" HPLC column, HR 5/5, available from Pharmacia LKB Biotechnology of Piscataway, NJ, and the drop-through fractions pooled, concentrated, and buffer exchanged into the starting buffer for hydroxylapatite, 10 mM sodium phosphate, pH 7.4, 0.01 mM CaCl₂ as described above. The concentrated pools were loaded onto a HPHT hydroxylapatite HPLC column available from Bio-Rad of Richmond, CA, and eluted with a linear gradient of 10-400 mM sodium phosphate pH 7.4, 10 mM CaCl₂. As seen in FIG. 1, absorbances at 220 nm, 30, 254 nm 31, and 280 nm 32 were measured simultaneously using a diode-array spectrophotometer, Hewlett-Packard #1040A, available from Hewlett-Packard Analytical Instruments of Avondale, PA. Final pools were made based on UV absorbency, 30, 31, 32, and inhibitory activity, 33.

The following characterization data represents specific results of factor J purified according to example 1. The factor J isolated is a protein with the following properties. FIG. 2 is an autoradiograph of unreduced, Lane 42 and reduced, Lane 43, ¹²⁵I-factor J, run on a 3-20% slab SDS-PAGE gel. The major bands of factor J had a mobility of 18,400 M_r, 44, which did not change with reduction 45. Repeated analysis of factor J revealed a molecular weight which varied from 18,000, 44, to 22,000, 45. This variation is inherent in this method. A second prominent band was at 200,000 M_r, 46. Molecular weight determination was based on the ¹⁴C labeled protein standards: myosin (200,000), phosphorylase b (92,500), bovine serum albumin (69,000) ovalbumin (46,000) carbonic anhydrase (30,000) and lysozyme (14,100). We believe the true molecular weight to be about 20,000 M_r because manipulations such as storage, heating, exposure to low pH or reducing agents increased the relative amounts of the 200,000 M_r, and decreased the relative amount of the 20,000 M_r form.

Isolated factor J has the capacity to agglutinate the erythrocytes of various species (human, rabbit, guinea pig and sheep erythrocytes have been tested, and all are positive). This agglutination becomes apparent after the factor J has passed through QAE-"SEPHADEX". The agglutination titer and functional inhibitory titers are roughly parallel. The agglutination can be inhibited by commercial heparin.

The amino acid composition of isolated human urine factor J revealed a relatively small amount of tyrosine, about 8.7 residues per 1000, which is consistent with the poor reactivity of factor J in Folin Assays. In addition, UV spectra of purified factor J, FIG. 3, suggests a low tryptophan value which is demonstrated by the minimal absorption of purified factor J at 280 nm, 55.

Results indicate that factor J is not an enzyme. Factor J inhibition occurs rapidly as can be seen in FIG. 4. Factor J reached maximum inhibitory potential within approximately five minutes, 61. Inhibitory potential was measured using a functional hemolytic assay. FIG. 5 shows that factor J activity inhibition is not affected by temperature. There was no significant change in activity at temperatures ranging from 40° C., 62, to 37° C., 65. FIG. 6 shows a reciprocal plot of the data which indicates that factor J inhibition is noncompetitive, 68. This suggests that the catalytic subunit and factor J are binding reversibly, randomly and independently at different sites. Accordingly factor J could be binding to C1q directly or it could be binding to C1q once its is bound to C1r₂s₂.

Factor J did inhibit association of the C1 complex as measured by factor J's ability to inhibit the precipitation of ¹²⁵I-C1q in the presence of C1r and C1s, FIG. 7. This titration profile, 71, was very similar to that obtained when the dose response of factor J inhibition of C1 formation in the hemolytic assay, 72. Both assays were measured over the same concentration range of polypeptide. The difference in the shape of the inhibition curves for ¹²⁵I-C1q interaction with C1r₂s₂, 71, and the inhibition of C1 hemolytic activity, 72, emphasizes that factor J inhibits the C1q and C1r₂s₂ reaction in a saturable manner consistent with direct binding to C1, whereas the inhibition of C1 hemolytic function follows a sigmoidal 72 response consistent with the complex kinetics of erythrocyte lysis induced by diluted serum.

Although factor J has been shown to inhibit C1q association with C1r₂s₂, the mechanisms for this inhibition are not the same as that of C1 inhibitor or C1q inhibitor. C1 inhibitor acts by binding to both catalytic subunits of C1, C1r and C1s. An assay measuring esterase activity of purified C1s, FIG. 8 compares factor J inhibition for C1s, 82, with inhibition of the C1 inhibitor for C1s, 83. As seen in FIG. 8, C1s in the presence of factor J, 82, or buffer alone, 81, show comparable amounts of esterase activity, whereas, the addition of C1 inhibitor resulted in a significant decrease in C1 esterase activity, 83. C1q inhibitor acts by binding to C1q and thereby preventing the catalytic subunits from binding to C1q. FIG. 9 demonstrates that factor J does not bind to C1q under conditions in which the C1q inhibitor could bind C1q. Partially purified C1q inhibitor bound to and precipitated ¹²⁵I-C1q, 101, whereas, purified factor J did not bind to ¹²⁵I-C1q in the fluid phase to permit precipitation of the ¹²⁵I-C1q, 102.

Factor J can also inhibit the human alternative complement pathway in an assay utilizing sheep erythrocytes bearing human C3b, and purified factor D, factor

B, and peiperidin. The process step in the alternative pathway where factor J inhibits is not yet known.

Antigenic results indicate that factor J is present in human serum and it does not cross react with antigen for C1 Inhibitor. FIG. 10 shows that goat anti-human serum precipitates radiolabeled factor J, 121, above the background level precipitated by normal goat serum, 122. Goat anti-C1q, 123, did not cause any precipitation of radiolabeled factor J above background. In addition, FIG. 11 demonstrates that factor J is not antigenically related to C1 Inhibitor. The anti-C1 Inhibitor did not specifically absorb the factor J, 111, as compared with anti-5, a control, 112, whereas the anti-C1 Inhibitor was able to specifically absorb ¹²⁵I-C1 Inhibitor under the same conditions, 113.

Having above indicated a preferred embodiment of the present invention it will occur to those skilled in the art that modifications and alternatives can be practiced within the spirit and scope of the invention. It is accordingly intended to define the scope of the invention only as indicated in the following claims.

EXAMPLE 2

Approximately 100-200 ml of serum is collected and saturated to 15% (weight/weight) with polyethylene glycol available from Sigma Chemical Co of St. Louis, MO. The saturated solution is kept at 4° C for 30 minutes and then centrifuged. The precipitate is collected and the supernatant is discarded.

The precipitate is solubilized with pH 7.5 NaCl phosphate buffer and further diluted with water to adjust the solution to a conductivity of about 4 mS. This adjusted solution is loaded onto a DEAE-"SEPHACEL" available from Pharmacia LKB Biotechnology of Piscataway, NJ, column (5×50 cm). The non-absorbed material is collected, pooled and adjusted to pH 9. The adjusted material is then applied to a QAE-"SEPHADEX" A-50 available from Pharmacia LKB Biotechnology of Piscataway, NJ, column (5×30 cm) equilibrated in 1 mM NaCl, 5 mM Tris, pH 9. The effluent is collected, pooled and adjusted to pH 7.2.

The adjusted effluent pool was loaded on a heparin-Sepharose column (5×15 cm) made from crude porcine heparin coupled by cyanogen bromide to "SEPHAROSE"-4B, available from Pharmacia LKB Biotechnology of Piscataway, NJ, and equilibrated in 25 mM NaCl, 50 mM Tris, pH 7.4. Factor J activity eluted between 18-20 mS during a linear gradient of starting buffer made with 1.5 M NaCl. Pools of fractions with factor J activity were concentrated and buffer exchanged by ultrafiltration using a cellulose 100 m, cut-off membrane, Spectra/Por type C, available from Spectrum Medical Industries of Los Angeles, CA, into the starting buffer for the "MONO-Q" column, 40 mM NaCl, 10 mM sodium phosphate, pH 7.4, 2 mM DMSA, 0.01% Na₂S₂O₃, 5 mM PMSF. The concentrated pools were loaded onto a "MONO Q" HPLC column, HR 5/5, available from Pharmacia LKB Biotechnology of Piscataway, NJ, and the drop-through fractions pooled, concentrated, and buffer exchanged into the starting buffer for the hydroxylapatite column, 10 mM sodium phosphate, pH 7.4, 0.01 mM CaCl₂. The concentrated pools were loaded onto a HPHT hydroxylapatite HPLC column available from Bio-Rad of Richmond, CA, and eluted with a linear gradient of 10-400 mM sodium phosphate pH 7.4, 10 mM CaCl₂. Pools were made based on UV absorbency and inhibitory activity. This pool is then applied to a Vydac C4 reverse phase

column, available from Vydac of Hisperia, CA, which has been equilibrated with 0.1% trifluoroacetic acid (TFA) in water. The factor J is eluted in a gradient made with the equilibration buffer and 95% acetonitrile in 5% water, 0.1% TFA. The elution rate is 1 ml/min and the factor J peak elutes at about 19 min in a 30 minute run. The fractions are dried down and reconstituted in 0.1 M NH_4HCO_3 buffer for assaying.

In practice, factor J is isolated, purified and administered in a therapeutic amount to inhibit the undesirable activation of the complement cascade system.

What is claimed is:

1. A method of inhibiting complement activation comprising:

administering purifying factor J with the following characteristics:

- a) elutes as a single peak from a hydroxylapatite column with symmetrical U.V. absorbance at 200, 250 and 280 nm;
- b) a tyrosine composition of about 8.7 residues per 1000 residues;

c) inhibits C1;

d) does not inhibit C1s;

e) dissociates intact C1;

f) presents C1 assembly form subcomponents

g) when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, migrates to a region corresponding to a molecular weight between 18,000 and 22,000 Daltons, whether reduced or non-reduced;

h) agglutinates the erythrocytes from several species of mammals including human, rabbit, guinea pig and sheep and this agglutination is inhibited by heparin;

i) inhibits the alternative complement pathway; and

j) is free of other known inhibitors of C1; in an amount effective to inhibit the undesirable activation of the complement cascade.

2. The method of claim 1 wherein said factor J is purified from urine collected from the patient.

3. The method of claim 1 wherein said factor J is purified from serum collected from the patient.

* * * * *

25

30

35

40

45

50

55

60

65

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,268,363

Page 1 of 4

DATED : December 7, 1993

INVENTOR(S) : Anne Nicholson-Weller

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 1, line 56, after "Another" delete "Inhibitor" and insert therefor --inhibitor--.

Col. 2, line 11, after ""SEPHADEX"" insert --,--.

Col. 2, line 12, after ""MONO Q"" insert --,--.

Col. 3, line 22, after "salt" insert --gradient. The factor J fractions are pooled and loaded--.

Col. 3, line 29, delete "MONO Q" and insert therefor --"MONO Q"--.

Col. 3, line 29, after "is" delete "and" and insert therefor --an--.

Col. 3, line 51, after "5" delete "MM" and insert therefor --mM--.

Col. 3, line 52, after "azide" delete "(NaN3);" and insert therefor --(NaN₃);--.

Col. 3, line 52, after "2" delete "mm" and insert therefor --mM--.

Col. 4, line 3, after "through" delete "fraction" and insert therefor --fractions--.

Col. 4, line 4, after "5", second occurrence, delete "MM" and insert therefor --mM--.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,268,363

Page 2 of 4

DATED : December 7, 1993

INVENTOR(S) : Anne Nicholson-Weller

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 4, line 7, after "5" delete "mm" and insert therefor --mM--.

Col. 4, line 11, after "500" delete "mm" and insert therefor --mM--.

Col. 4, line 21, after "25" delete "mm" and insert therefor --mM--.

Col. 4, line 21, after "50" delete "mm" and insert therefor --mM--.

Col. 4, line 30, after "2" delete "mm" and insert therefor --mM--.

Col. 4, line 30, after "0,01%" delete "NaN3," and insert therefor --NaN₃--.

Col. 4, line 36, before "sodium" delete "mm" and insert therefor --mM--.

Col. 4, line 36, after "0.01" delete "mm" and insert therefor --mM--.

Col. 4, line 41, after "10" delete "um CaCl₂," and insert therefore --uM CaCl₂--.

Col. 5, line 13, before "spectra" delete "Uv" and insert therefor --UV--.

Col. 5, line 21, after "activity" insert --(% --.

Col. 5, line 23, after "from" delete "40°" and insert therefor --4°--.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,268,363

Page 3 of 4

DATED : December 7, 1993

INVENTOR(S) : Anne Nicholson-Weller

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 6, line 43, before "column" delete "Sepharose" and insert therefor --"SEPHAROSE"--.

Col. 6, line 51, after "100" delete "m_r" and insert therefor --M_r--.

Col. 6, line 61, after "10" delete "mm" and insert therefor --mM--.

Col. 6, line 66, after "10" delete "um" and insert therefor --uM--.

Col. 7, line 8, after "0.1 M" delete "NH₄HCO₃" and insert therefor --NH₄HCO₃--.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,268,363

Page 4 of 4

DATED : December 7, 1993

INVENTOR(S) : Anne Nicholson-Weller

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 7, line 15, after "administering" delete "purifying" and insert therefor --purified--.

Col. 7, line 18, after "at" delete "200," and insert therefor --220,--.

Col. 8, line 4, after "f)" delete "presents" and insert therefor --prevents--.

Col. 8, line 15, after "C1;" insert --to a patient in need thereof--.

Signed and Sealed this

Fourteenth Day of June, 1994

Attest:



BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

DOCUMENT-IDENTIFIER: US 5109114 A

TITLE: Characterization and method of isolation for an inhibitor of complement C1

BSPR:

The activation of the complement cascade can also cause undesirable phenomena, such as inflammation, damage of normal tissue and disease states such as the autoimmune diseases. Autoimmune diseases are associated with the immune

complexes formed against indigenous tissue which are associated with the biologically active complement fragments generated by the classical portion of the complement cascade. Such diseases include but are not limited to:

Hashimoto's thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, Graves' disease, myasthenia gravis, insulin resistance, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, and rheumatoid arthritis.

ORPL:

Ghebrehiet, "C1q Inhibitor (C1qINH): Functional Properties and Possible Relationship to a Lymphocyte Membrane-Associated C1q Precipitin", 126 J. Immunol. 1837-1842 (1981).



US005109114A

United States Patent [19][11] **Patent Number:** **5,109,114****Nicholson-Weller**[45] **Date of Patent:** **Apr. 28, 1992**[54] **CHARACTERIZATION AND METHOD OF ISOLATION FOR AN INHIBITOR OF COMPLEMENT C1**[75] **Inventor:** Anne Nicholson-Weller, Wellesley, Mass.[73] **Assignee:** Beth Israel Hospital, Boston, Mass.[21] **Appl. No.:** 406,144[22] **Filed:** Sep. 12, 1989[51] **Int. Cl.⁵** C07K 15/06[52] **U.S. Cl.** 530/350; 530/380[58] **Field of Search** 530/380, 350[56] **References Cited****U.S. PATENT DOCUMENTS**

4,147,801	4/1979	Lenhard et al.	424/315
4,388,232	6/1983	Eibl et al.	530/380
4,883,784	11/1989	Kaneko	514/8
4,915,945	4/1990	Pelzer et al.	421/101

OTHER PUBLICATIONS

Lopez-Trascaso et al. 1989, J. Biol. Chem. 264(27): 16214-16221.

Abstract, Biosis Number 62064952 of Cheung et al., 1986, Biochem Biophys Acta, 882(2): 200-209.

Shebrehwet et al., 1988, J. Immunol. Methods, 110: 251-260.

Silvestri et al., 1981, J. Biol. Chem. 256(14): 7383-7387.

Cooper, "The Classical Complement Pathway: Activation and Regulation of the First Complement Component", 37 Adv. Immunol 151-216 (1985).

Schumaker et al., "Activation of the First Component of Complement", 5 Ann. Rev. Immunol. 21-42 (1987).
 Reid et al., "The Proteolytic Activation Systems of Complement", 50 Ann. Rev. Biochem. 433-464 (1981).
 Ziccardi, "The Role of Immune Complexes in the Activation of the First Component of Human Complement", 132 J. Immunol. 283-288 (1984).
 Ziccardi, "Demonstration of the Interaction of Native C1 with Monomeric Immunoglobulins and C1 Inhibitor", 134, J. Immunol, 2559-2563 (1985).
 Ghebrehiet, "C1q Inhibitor (C1qINH): Functional Properties and Possible Relationship to a Lymphocyte Membrane-Associated C1q Precipitin", 126 J. Immunol. 1837-1842 (1981).

Ghebrehwet, "Purification and Partial Characterization of a C1q Inhibitor from the Membranes of Human Peripheral Blood Lymphocytes", 129 J. Immunol. 151-162 (1982).

Davis, "C1 Inhibitor and Hereditary Angioneurotic Edema", 6, Ann. Rev. Immunol. 595-628 (1988).

Primary Examiner—Margaret Moskowitz*Assistant Examiner*—Keith C. Furman*Attorney, Agent, or Firm*—Lorusso & Loud

[57]

ABSTRACT

A purified protein, factor J, which has inhibitory properties which prevent the formation or the dissociation of C1 complex and a method of purification for said protein. The method including the following sequential chromatography steps: anion exchange, QAE-Sephadex, heparin-Sepharose affinity, Mono-Q and hydroxylapatite.

3 Claims, 6 Drawing Sheets

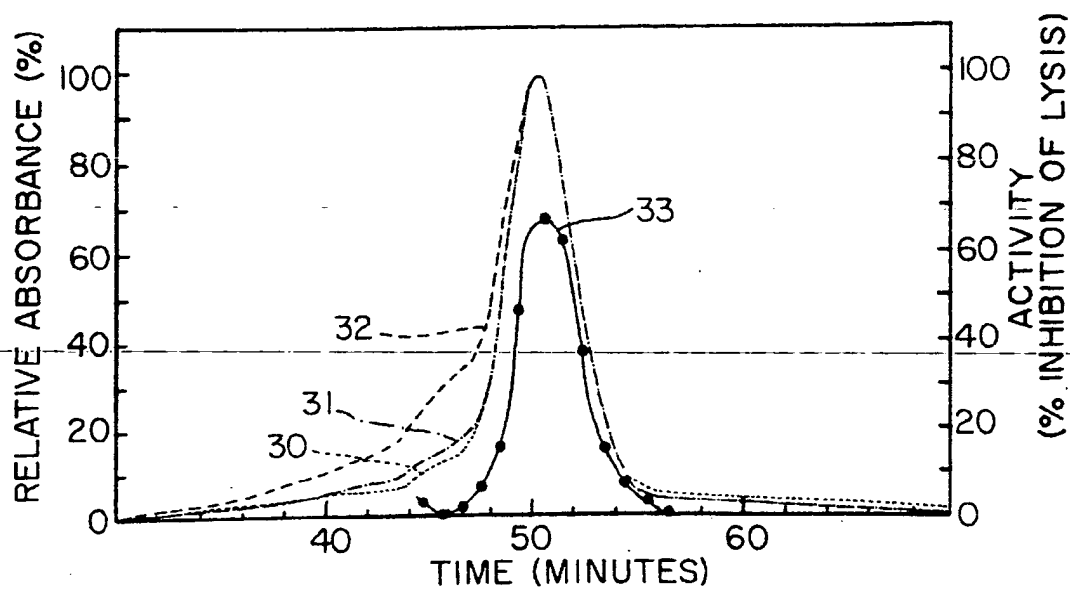


FIG. 1

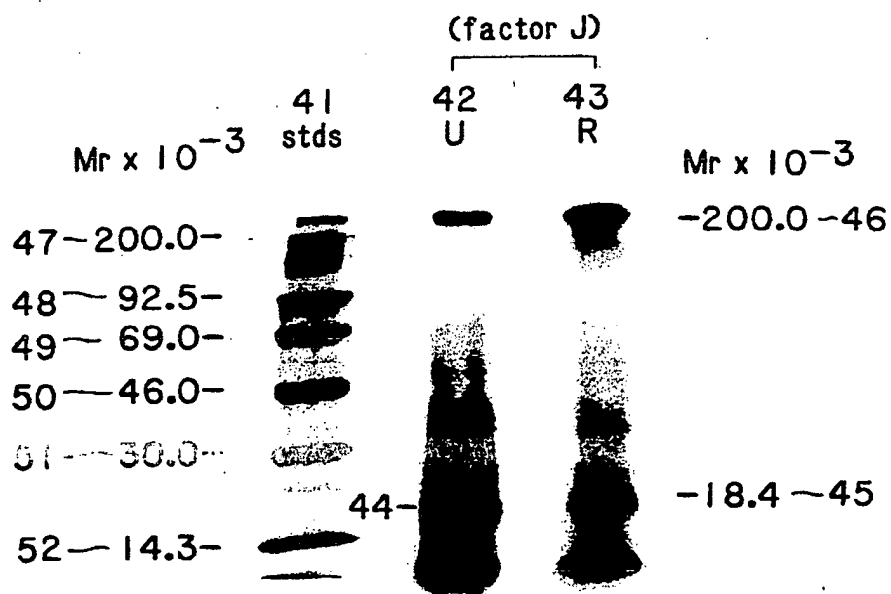


FIG. 2

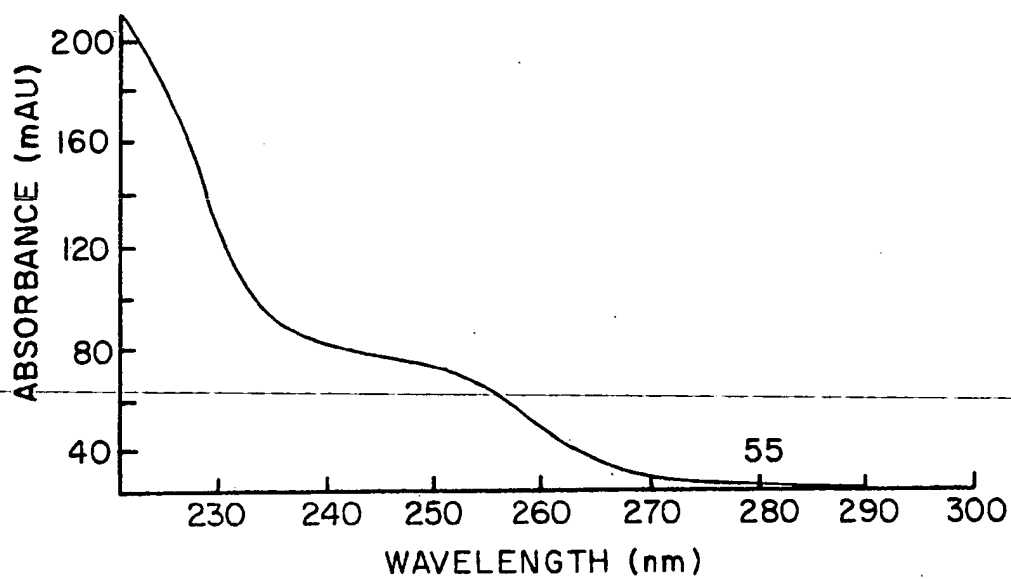
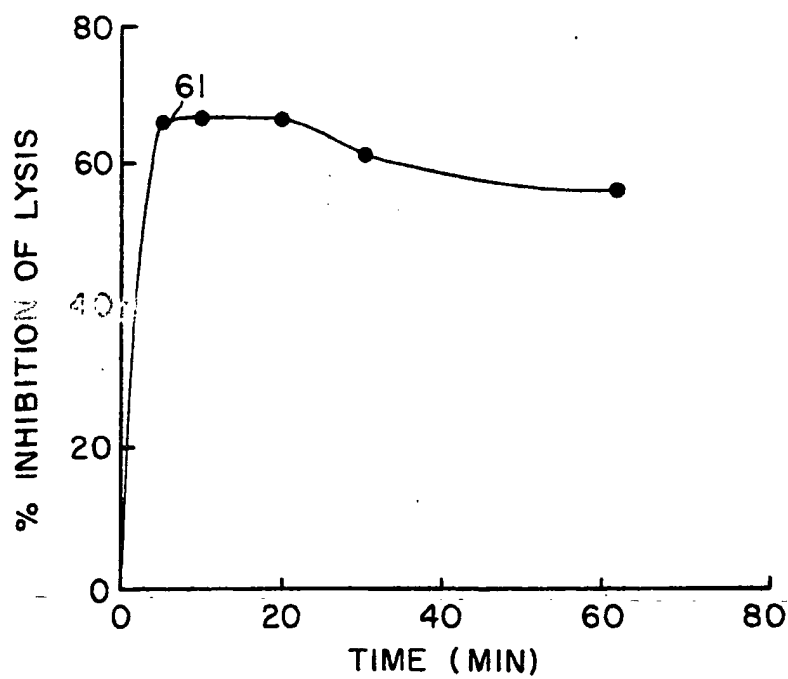


FIG. 3

FIG. 4



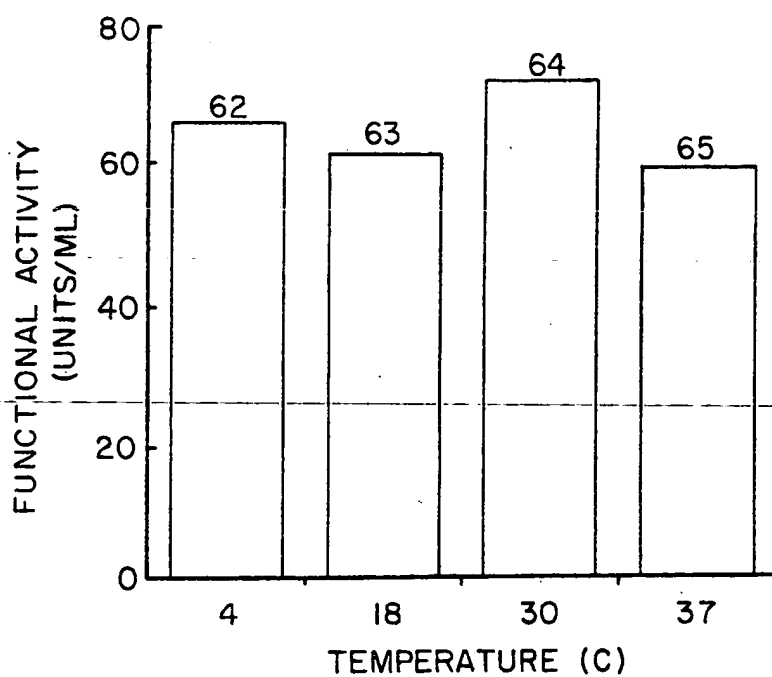
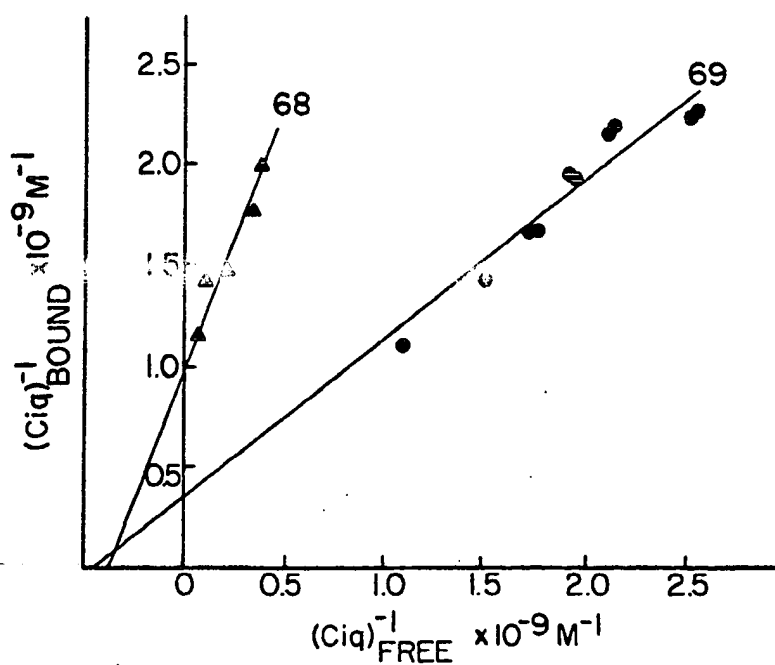


FIG. 5

FIG. 6



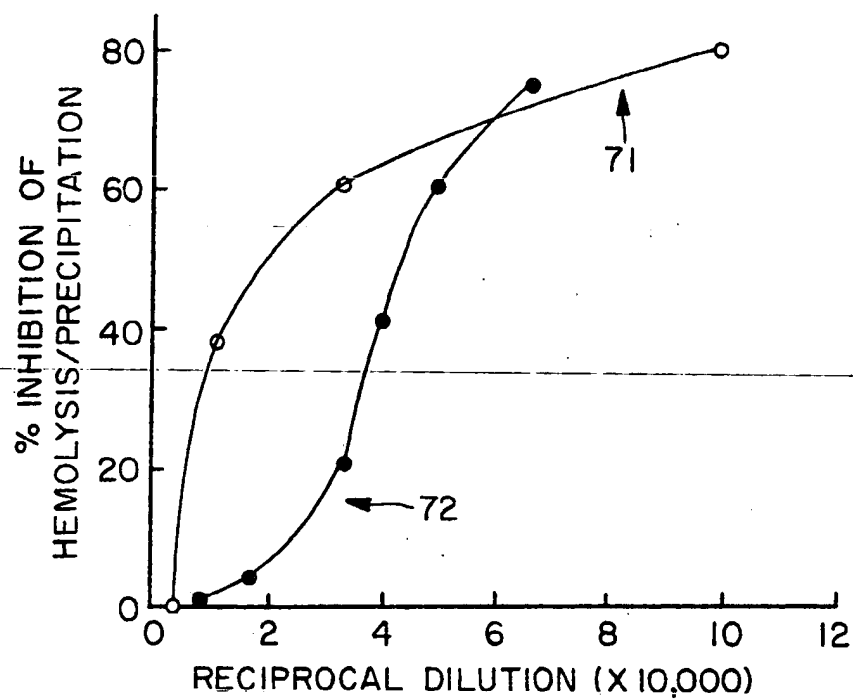
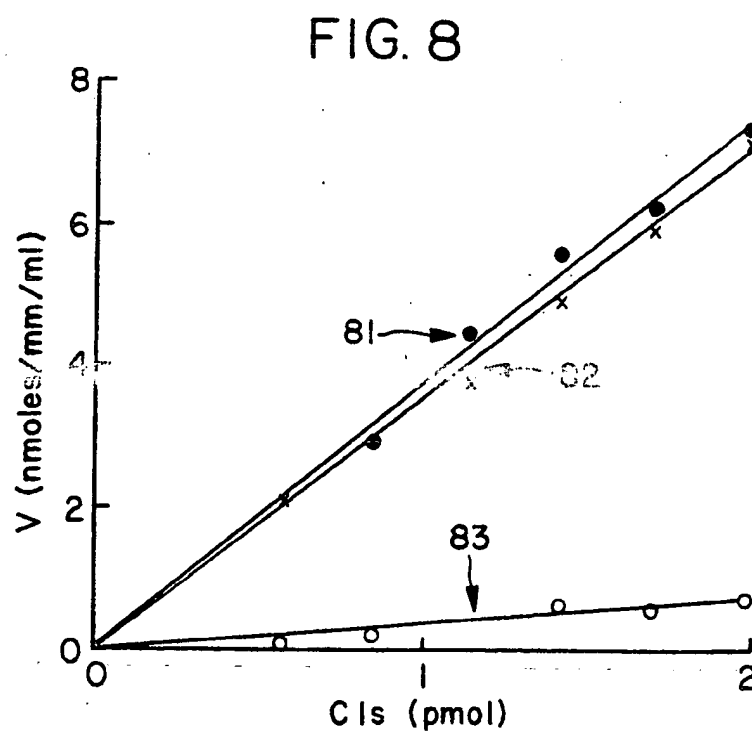


FIG. 7



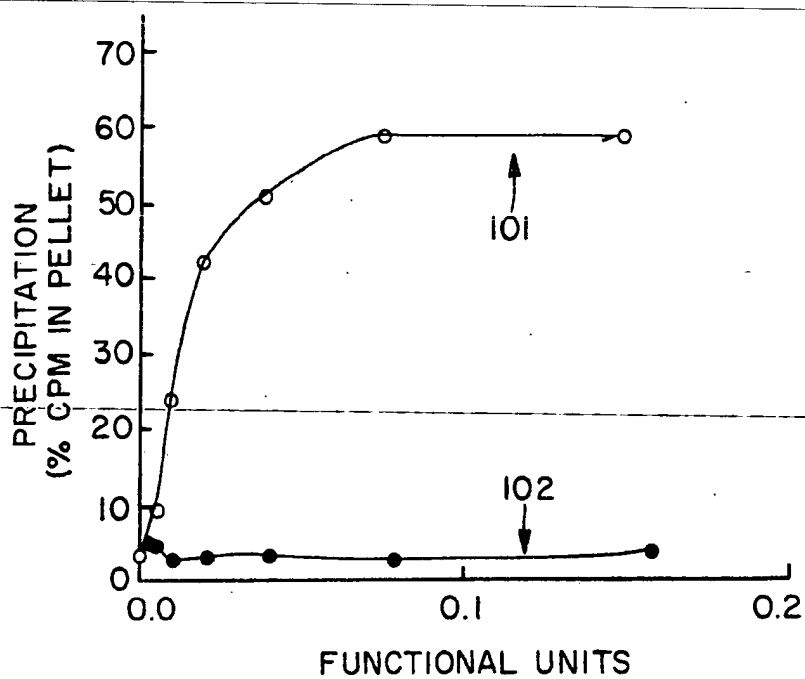


FIG. 9

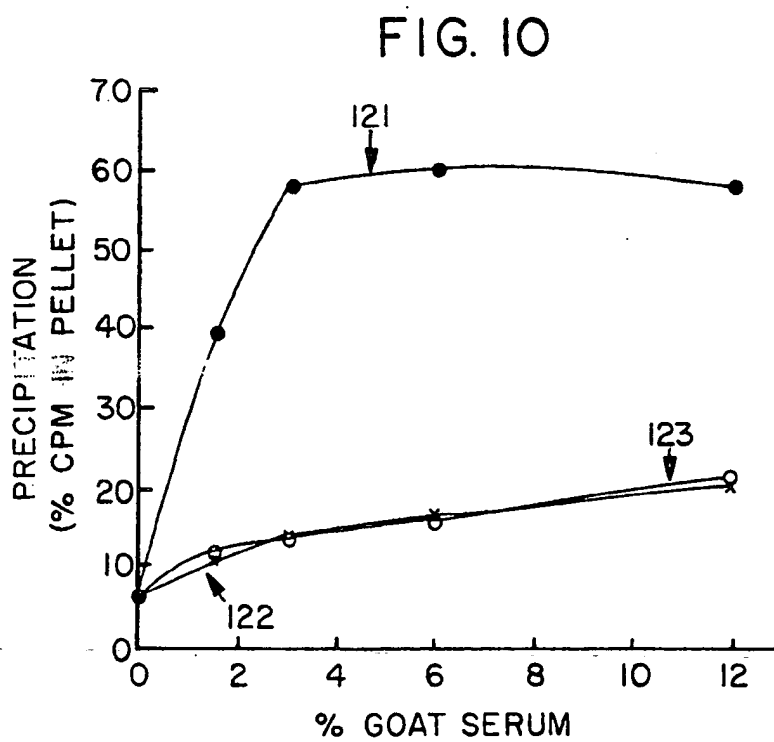
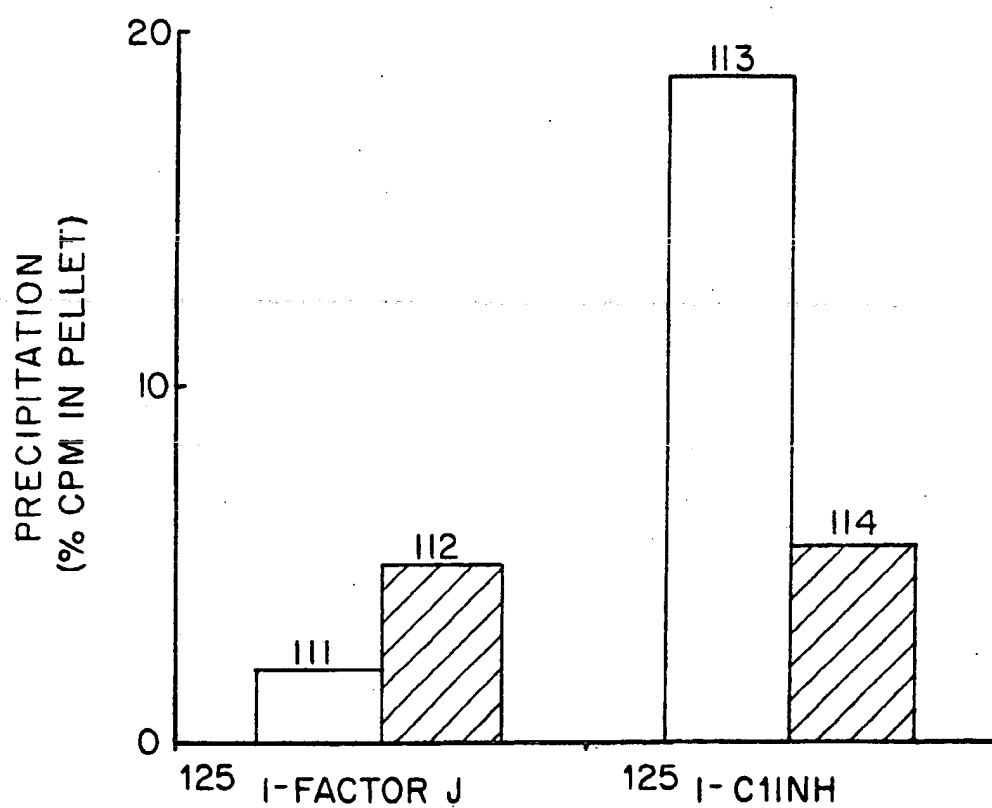


FIG. 10

FIG. II



CHARACTERIZATION AND METHOD OF ISOLATION FOR AN INHIBITOR OF COMPLEMENT C1

BACKGROUND OF THE INVENTION

The invention described herein was made with Government support and the U.S. Government has certain rights in the invention.

The immune system is the power of the body to resist invasion by pathogenic organisms, and to overcome such invasion and its ensuing infection, once it has taken place. The complement system is important in the immune response. Complement is a physiological process which involves many plasma proteins that react in a cascading (sequential) effect to mediate a number of desirable biologically-significant phenomena. Such phenomena include modulation of the immune response, facilitation of the transport of immune complexes, production of anaphylatoxins which cause release of histamine, chemotaxis which is the migration of cells towards the area of complement activity, phagocytosis, and lysis of cells.

The activation of the complement cascade can also cause undesirable phenomena, such as inflammation, damage of normal tissue and disease states such as the autoimmune diseases. Autoimmune diseases are associated with the immune complexes formed against indigenous tissue which are associated with the biologically active complement fragments generated by the classical portion of the complement cascade. Such diseases include but are not limited to: Hashimoto's thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, Graves' disease, myasthenia gravis, insulin resistance, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, and rheumatoid arthritis.

It is known that the first phase of complement activation begins with C1. C1 is made up of three distinct proteins: a recognition subunit, C1q, and the serine proteinase subcomponents, C1r and C1s which are bound together in a calcium-dependent tetrameric complex, C1r₂s₂. An intact C1 complex is necessary for physiological activation of C1 to result. Activation occurs when the intact C1 complex binds to immunoglobulin complexed with antigen. This binding activates C1s which would then react with the next plasma protein, C4, to start the cascading effect rolling.

In terms of the regulation of the complement system, most studies have focused on the binding properties of the C1 serine proteinase subcomponents, C1r and C1s, for a serum glycoprotein, C1 Inhibitor. Another inhibitor that has been identified but whose role in regulating C1 function in plasma is not clear is the C1q inhibitor (C1qINH).

It is important to identify and isolate inhibitors of the complement system because by isolating an inhibitor one may be able to control the effects of diseases such as those stated above. The inhibitors may provide a basis for pharmacologic intervention, either by allowing manipulation of the level of an inhibitor, or by providing a model for the chemical synthesis of a new inhibitor.

It is therefore an object of the present invention to provide a method for the isolation of an inhibitor of C1 which is functionally and antigenically distinct from known inhibitors of C1.

It is more specifically an object of the present invention to characterize the properties of an inhibitor of C1, factor J.

SUMMARY OF THE INVENTION

In accordance with the present invention, factor J is isolated from body fluid, in a multi-column purification procedure. The sequence of columns necessary for purification is an anion exchange, QAE-Sephadex, affinity, Mono Q, and hydroxylapatite HPLC columns. The purified Factor J has a molecular weight (Mr) of about 20,000 daltons, minimal absorption at 280 nm, and a relatively small number of tyrosine residues. The newly discovered protein has been found to inhibit the association of the tetrameric complex C1r₂s₂ with the recognition subunit C1q, and it can dissociate the fully assembled-activated C1 complex.

This inhibitor is functionally and antigenically distinct from other known inhibitors of C1, namely, C1INH and C1qINH. The inhibitory capabilities of C1 Inhibitor are the result of its binding to the catalytic subunits of C1, C1r and C1s, and thereby inhibiting C1r and C1s. The C1q Inhibitor can only inhibit the assembly of the C1 complex by prior binding to C1q. In contradistinction, it has been discovered that factor J does not inhibit C1s, and that factor J can both dissociate intact C1 as well as prevent its assembly from subcomponents. Thus, factor J is functionally distinct from C1 Inhibitor and C1q Inhibitor.

In its broadest overall aspect, factor J is first isolated and purified and then administered in a therapeutic amount to inhibit the undesirable activation of the complement cascade.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an elution profile of the purified factor J of the present invention, at three different simultaneously recorded wavelengths (A 220, 254, and 280 nm).

FIG. 2 is an autoradiograph of radiolabeled factor J of the present invention, after being run on a SDS-PAGE 3-20% slab gel.

FIG. 3 is the UV spectra from 220-300 nm of the peak of factor J of the present invention, eluted from the hydroxylapatite column.

FIG. 4 is a graph showing the kinetics of factor J activity of the present invention as plotted on the y axis against the incubation time.

FIG. 5 is a bar graph which plots the temperature at which a hemolytic assay was performed on the x axis and the activity of factor J of the present invention (% inhibition of lysis using a hemolytic assay) on the y axis.

FIG. 6 is a reciprocal plot showing factor J of the present invention is a noncompetitive inhibitor.

FIG. 7 is a graph plotting the dose response of factor J of the present invention when titered, x axis, in the hemolytic and C1 complex formation assays.

FIG. 8 is a graph showing the inability of factor J of the present invention to bind to the catalytic subunit of C1 as compared to C1 Inhibitor.

FIG. 9 is a graph of the addition of comparable amounts of functional activity of C1qINH or factor J of the present invention (x axis) plotted against the amount of bound complex ¹²⁵I-C1 that is precipitable.

FIG. 10 is a graph indicating the presence of the factor J antigen of the present invention in human serum.

FIG. 11 is a bar graph showing the antigenic difference between CI Inhibitor and factor J of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery of a new, functionally and antigenically distinct inhibitor of CI complex association, factor J. A method is described for purifying and characterizing factor J.

The preparation starts with a sample of body fluid, such as, but not limited to, urine and serum, which has been dialyzed. The dialyzate is filtered and loaded onto an anion exchange column which has been equilibrated with dialysis buffer containing salt. The drop through fractions are collected and pooled.

The pooled fraction is diluted with the starting buffer of the QAE-Sephadex A-50 column and loaded onto the column. Factor J is collected in the drop through and early eluted fractions of the linear salt gradient. The factor J fractions are pooled and loaded onto a heparin-Sepharose affinity column. Factor J elutes between 18 and 20 mS during a linear salt gradient when the column is equilibrated at pH 7.4 and NaCl provides the counter ion. Pools of fractions with factor J are concentrated and the buffer exchanged for the starting buffer of the Mono-Q column with inhibitors.

The concentrated solution is loaded on the Mono Q column and the drop through fractions are pooled, concentrated and the buffer exchanged with phosphate starting buffer of the hydroxylapatite column. The solution is then loaded onto a hydroxylapatite column and eluted with an increasing linear phosphate gradient. Absorbances at 220, 250 and 280 are measured and the final pools are made based on the UV absorbency and inhibitory activity.

The following example is submitted to illustrate but not limit this invention.

EXAMPLE 1

Human urine was collected from normal donors in 250ml polypropylene bottles containing stock amounts of the following inhibitors calculated to achieve the following final concentrations: 1 mM phenylmethylsulfonyl fluoride (PMSF); 5 mM EDTA; 0.01% sodium azide (NaN₃); 1 ug/ml leupeptin; 2 mM benzamide-HCl; 1 ug/ml aprotinin. Upon collection of 250 ml of urine the bottle was frozen immediately at -70° C. To initiate the purification procedure the requisite number of bottles to provide 800-1000 ml urine were thawed, the urine adjusted to pH 7.4 with a saturated solution of Na₂HPO₄, and dialyzed in 3,500 M, cut-off tubing against 4 changes of 10 liters of 10 mM sodium phosphate, pH 7.4, 2 mM EDTA, 0.01% NaN₃, 0.5 mM PMSF buffer until the conductivity was 2 mS, or less. The dialyzed urine was filtered through a 3 um pore polypropylene filter, available from Pall-Chisholm Company of Cranston, RI, and loaded onto a DEAE-Sephacel, available from Pharmacia LKB Biotechnology of Piscataway, N.J., column (5×50 cm) equilibrated in the dialysis buffer with 0.04 M NaCl added. The drop-through fractions were pooled and the pool diluted with 5 volumes of 5 mM Tris buffer, pH 9, and applied to a QAE-Sephadex A-50, available from Pharmacia LKB Biotechnology of Piscataway, N.J., column (5×30 cm) equilibrated in 1 mM NaCl, 5 mM Tris, pH 9. Factor J activity was about equally present in the drop-through fractions and in the early eluted fractions

when a linear gradient was applied of starting buffer made with 500 mM NaCl.

Separate pools of factor J were made from the drop-through and eluted fractions, and these pools were kept separate over the subsequent purification steps, although subsequent studies indicated there was no detectable difference in the factor J from the two pools. Each pool was loaded on a heparin-Sepharose column (5×15 cm) made from crude porcine heparin coupled by cyanogen bromide to Sepharose-4B available from Pharmacia LKB Biotechnology of Piscataway, N.J., and equilibrated in 25 mM NaCl, 50 mM Tris, pH 7.4. Factor J activity eluted between 18-20 mS during a linear gradient of starting buffer made with 1.5 M NaCl. Pools of fractions with factor J activity were concentrated and buffer exchanged by ultrafiltration using a cellulose 1000 M, cut-off membrane, Spectra/Por type C, available from Spectrum Medical Industries of Los Angeles, CA, into the starting buffer for the Mono Q, 40 mM NaCl, 10 mM sodium phosphate, pH 7.8, 2 mM EDTA, 0.01% NaN₃, 5 mM PMSF. The concentrated pools were loaded onto a Mono Q HPLC column, HR 5/5, available from Pharmacia LKB Biotechnology of Piscataway, N.J., and the drop-through fractions pooled, concentrated, and buffer exchanged into the starting buffer for hydroxylapatite, 10 mM sodium phosphate, pH 7.4, 0.01 mM CaCl₂ as described above. The concentrated pools were loaded onto a HPHT hydroxylapatite HPLC column available from Bio-Rad of Richmond, CA, and eluted with a linear gradient of 10-400 mM sodium phosphate pH 7.4, 10 mM CaCl₂. As seen in FIG. 1, absorbances at 220nm, 30, 254nm 31, and 280 nm 32 were measured simultaneously using a diode-array spectrophotometer, Hewlett-Packard #1040A, available from Hewlett-Packard Analytical Instruments of Avondale, PA. Final pools were made based on UV absorbency, 30, 31, 32, and inhibitory activity, 33.

The following characterization data represents specific results of factor J purified according to example 1.

The factor J isolated is a protein with the following properties. FIG. 2 is an autoradiograph of unreduced, Lane 42 and reduced, Lane 43, ¹²⁵I-factor J, run on a 3-20% slab SDS-PAGE gel. The major bands of factor J had a mobility of 18,400 M_r, 44, which did not change with reduction 45. Repeated analysis of factor J revealed a molecular weight which varied from 18,000, 44, to 22,000, 45. This variation is inherent in this method. A second prominent band was at 200,000 M_r, 46. Molecular weight determination was based on the ¹⁴C labeled protein standards: myosin (200,000), phosphorylase b (92,500), bovine serum albumin (69,000) ovalbumin (46,000) carbonic anhydrase (30,000) and lysozyme (14,100). We believe the true molecular weight to be about 20,000 M_r, because manipulations such as storage, heating, exposure to low pH or reducing agents increased the relative amounts of the 200,000 M_r and decreased the relative amount of the 20,000 M_r form.

Isolated factor J has the capacity to agglutinate the erythrocytes of various species (human, rabbit, guinea pig and sheep erythrocytes have been tested, and all are positive). This agglutination becomes apparent after the factor J has passed through QAE-Sephadex. The agglutination titer and functional inhibitory titers are roughly parallel. The agglutination can be inhibited by commercial heparin.

The amino acid composition of isolated human urine factor J revealed a relatively small amount of tyrosine,

about 8.7 residues per 1000, which is consistent with the poor reactivity of factor J in Folin Assays. In addition, UV spectra of purified factor J, FIG. 3, suggests a low tryptophan value which is demonstrated by the minimal absorption of purified factor J at 280nm, 55.

Results indicate that factor J is not an enzyme. Factor J inhibition occurs rapidly as can be seen in FIG. 4. Factor J reached maximum inhibitory potential within approximately five minutes, 61. Inhibitory potential was measured using a functional hemolytic assay. FIG. 5 shows that factor J activity (% inhibition) is not affected by temperature. There was no significant change in activity at temperatures ranging from 4° C., 62, to 37° C., 65. FIG. 6 shows a reciprocal plot of the data which indicates that factor J inhibition is noncompetitive, 68. This suggests that the catalytic subunit and factor J are binding reversibly, randomly and independently at different sites. Accordingly factor J could be binding to Clq directly or it could be binding to Clq once its is bound to Clr₂s₂.

Factor J did inhibit association of the Cl complex as measured by factor J's ability to inhibit the precipitation of ¹²⁵I-Clq in the presence of Clr and Cls, FIG. 7. This titration profile, 71, was very similar to that obtained when the dose response of factor J inhibition of Cl formation in the hemolytic assay, 72. Both assays were measured over the same concentration range of polypeptide. The difference in the shape of the inhibition curves for ¹²⁵I-Clq interaction with Clr₂s₂, 71, and the inhibition of Cl hemolytic activity, 72, emphasizes that factor J inhibits the Clq and Clr₂s₂ reaction in a saturable manner consistent with direct binding to Cl, whereas the inhibition of Cl hemolytic function follows a sigmoidal 72 response consistent with the complex kinetics of erythrocyte lysis induced by diluted serum. 75

Although factor J has been shown to inhibit Clq association with Clr₂s₂, the mechanisms for this inhibition are not the same as that of Cl inhibitor or Clq inhibitor. Cl inhibitor acts by binding to both catalytic subunits of Cl, Clr and Cls. An assay measuring esterase activity of purified Cls, FIG. 8 compares factor J inhibition for Cls, 82, with inhibition of the Cl inhibitor for Cls, 83. As seen in FIG. 8, Cls in the presence of factor J, 82, or buffer alone, 81, show comparable amounts of esterase activity, whereas, the addition of Cl Inhibitor resulted in a significant decrease in Cl esterase activity, 83. Clq inhibitor acts by binding to Clq and thereby preventing the catalytic subunits from binding to Clq. FIG. 9 demonstrates that factor J does not bind to Clq under conditions in which the Clq Inhibitor could bind to Clq. Partially purified Clq inhibitor bound to and precipitated ¹²⁵I-Clq, 101, whereas, purified factor J did not bind to ¹²⁵I-Clq in the fluid phase to permit precipitation of the ¹²⁵I-Clq, 102.

Factor J can also inhibit the human alternative complement pathway in an assay utilizing sheep erythrocytes bearing human C3b, and purified factor D, factor B, and peperidin. The process step in the alternative pathway where factor J inhibits is not yet known.

Antigenic results indicate that factor J is present in human serum and it does not cross react with antigen for Cl Inhibitor. FIG. 10 shows that goat anti-human serum precipitates radiolabeled factor J, 121, above the background level precipitated by normal goat serum, 122. Goat anti-Clq, 123, did not cause any precipitation of radiolabeled factor J above background. In addition, FIG. 11 demonstrates that factor J is not antigenically related to Cl Inhibitor. The anti-Cl Inhibitor did not

specifically absorb the factor J, 111, as compared with anti-5, a control, 112, whereas the anti-Cl Inhibitor was able to specifically absorb ¹²⁵I-Cl Inhibitor under the same conditions, 113.

Having above indicated a preferred embodiment of the present invention it will occur to those skilled in the art that modifications and alternatives can be practiced within the spirit and scope of the invention. It is accordingly intended to define the scope of the invention only as indicated in the following claims.

EXAMPLE 2

Approximately 100-200 ml of serum is collected and saturated to 15% (weight/weight) with polyethylene glycol available from Sigma Chemical Co of St. Louis, MO. The saturated solution is kept at 4° C. for 30 minutes and then centrifuged. The precipitate is collected and the supernatant is discarded.

The precipitate is solubilized with pH 7.5 NaCl phosphate buffer and further diluted with water to adjust the solution to a conductivity of about 4 mS. This adjusted solution is loaded onto a DEAE-Sephacel, available from Pharmacia LKB Biotechnology of Piscataway, N.J., column (5×50 cm). The non-absorbed material is collected, pooled and adjusted to pH 9. The adjusted material is then applied to a QAE-Sephadex A-50, available from Pharmacia LKB Biotechnology of Piscataway, N.J., column (5×30 cm) equilibrated in 1 mM NaCl, 5 mM Tris, pH 9. The effluent is collected, pooled and adjusted to pH 7.2.

The adjusted effluent pool was loaded on a heparin-Sepharose column (5×15 cm) made from crude porcine heparin coupled by cyanogen bromide to Sepharose-4B, available from Pharmacia LKB Biotechnology of Piscataway, N.J., and equilibrated in 25 mM NaCl, 50mM Tris, pH 7.4. Factor J activity eluted between 18-20 mS during a linear gradient of starting buffer made with 1.5 M NaCl. Pools of fractions with factor J activity were concentrated and buffer exchanged by ultrafiltration using a cellulose 100 M, cut-off membrane, Spectra/Por type C, available from Spectrum Medical Industries of Los Angeles, CA, into the starting buffer for the Mono Q column, 40 mM NaCl, 10 mM sodium phosphate, pH 7.8, 2 mM EDTA, 0.01% NaN₃, 5 mM PMSF. The concentrated pools were loaded onto a Mono Q HPLC column, HR 5/5, available from Pharmacia LKB Biotechnology of Piscataway, N.J., and the drop-through fractions pooled, concentrated, and buffer exchanged into the starting buffer for the hydroxylapatite column, 10 mM sodium phosphate, pH 7.4, 0.01 mM CaCl₂. The concentrated pools were loaded onto a HPHT hydroxylapatite HPLC column available from Bio-Rad of Richmond, CA, and eluted with a linear gradient of 10-400 mM sodium phosphate pH 7.4, 10 mM CaCl₂. Pools were made based on UV absorbency and inhibitory activity. This pool is then applied to a Vydac C4 reverse phase column, available from Vydac of Hisperia, CA, which has been equilibrated with 0.1% trifluoroacetic acid (TFA) in water. The factor J is eluted in a gradient made with the equilibration buffer and 95% acetonitrile in 5% water, 0.1% TFA. The elution rate is 1ml/min and the factor J peak elutes at about 19 min in a 30 minute run. The fractions are dried down and reconstituted in 0.1 M NH₄HCO₃ buffer for assaying.

In practice, factor J is isolated, purified and administered in a therapeutic amount to inhibit the undesirable activation of the complement cascade system.

What is claimed is:

1. A purified and isolated factor J with the following characteristics:

- a) elutes as a single peak from a hydroxylapatite column with symmetrical U.V. absorbance at 220, 250 and 280 nm;
- b) a tyrosine composition of about 8.7 residues per 1000 residues;
- c) inhibits Cl;
- d) does not inhibit Cls;
- e) dissociates intact Cl;
- f) prevents Cl assembly from subcomponents;
- g) when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, migrates to a region corresponding to a molecular weight between

18,000 and 22,000 Daltons, whether reduced or non-reduced;

- h) agglutinates the erythrocytes from several species of mammals including human, rabbit, guinea pig and sheep and this agglutination is inhibited by heparin;

- i) inhibits the alternative complement pathway; and
- j) is free of other known inhibitors of Cl.

2. The purified inhibitor of claim 1 wherein said inhibitor, factor J, is derived from a body fluid.

3. The purified inhibitor, factor J, of claim 2 wherein said body fluid is urine.

* * * * *

STIC-ILL

349194

From: Romeo, David
Sent: Monday, June 04, 2001 12:48 PM
To: STIC-ILL
Subject: 09503421 Klickstein L B; Barbashov S F; Liu T; Jack R M; Nicholson-Weller A

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

3347176

PLEASE COPY REFERENCE.

AU Klickstein L B; Barbashov S F; Liu T; Jack R M; Nicholson-Weller A
TI Complement receptor type 1 (CR1, CD35) is a receptor for
Clq.
SO IMMUNITY, (1997 Sep) 7 (3) 345-55.
Journal code: CCF; 9432918. ISSN: 1074-7613.
AN 97464534

4.20/4

Complement Receptor Type 1 (CR1, CD35) Is a Receptor for C1q

Lloyd B. Klickstein,^{††} Sergei F. Barbash,^{v,‡}
Tun Liu,^{‡‡} Richard M. Jack,[§]
and Anne Nicholson-Weller^{*‡}

^{*}Department of Medicine
Harvard Medical School

[†]The Division of Rheumatology/Immunology
Department of Medicine

Brigham and Women's Hospital

[‡]Charles A. Dana Research Institute

Harvard-Thorndike Laboratory

Division of Infectious Disease

Beth Israel-Deaconess Medical Center

Boston, Massachusetts 02215

[§]La Jolla Pharmaceutical

San Diego, California 92121

Summary

Molecular definition of the cellular receptor for the collagen domain of C1q has been elusive. We now report that C1q binds specifically to human CR1 (CD35), the leukocyte C3b/C4b receptor, and the receptor on erythrocytes for opsonized immune complexes. Biotinylated or radioiodinated C1q (^{*}C1q) bound specifically to transfected K562 cells expressing cell surface CR1 and to immobilized recombinant soluble CR1 (rsCR1). ^{*}C1q binding to rsCR1 was completely inhibited by unlabeled C1q and the collagen domain of C1q and was partially inhibited by C3b dimers. Kinetic analysis in physiologic saline of the interaction of unlabeled C1q with immobilized rsCR1 using surface plasmon resonance yielded an apparent equilibrium dissociation constant (K_{eq}) of 3.9 nM. Thus, CR1 is a cellular C1q receptor that recognizes all three complement opsonins, namely, C1q, C3b, and C4b.

Introduction

Complement C1q is the 462 kDa recognition subunit of multimeric C1, which when bound to a complement activating surface, causes the sequential activation of the enzymatic subunits of C1, C1r, and C1s. C1q avidly binds IgG and IgM in immune complexes and thereby "complements" acquired immunity by recruiting the classical complement pathway. In addition, C1q can directly bind DNA (Uwatoko et al., 1990), complexes of C-reactive protein (Jiang et al., 1991), serum amyloid P (Ying et al., 1993), isolated myelin (Vanguri et al., 1982), and urate crystals (Terkeltaub et al., 1983), as well as some gram-negative (Tenner et al., 1984) and gram-positive bacteria (Baker et al., 1982). By directly binding foreign or abnormally expressed host molecules and activating the classical pathway, C1q functions as part of the innate immune system. Whether activated by the acquired or innate immune system, the catalytic function

of C1 is tightly regulated by the C1 inhibitor, which covalently binds to the activated enzymatic subunits of C1, C1r, and C1s and removes them from C1q. C1q, still bound to its immune complex or activating substance via its globular domains, is now free to interact with cells bearing receptors for its collagen-like domain. The collagen-like domain, or collagen tail, is what remains of the C1q molecule after the six globular domains are digested away by pepsin (Reid, 1976). The collagen tail consists of the six "stems" of C1q, which come together in the amino half of the constituent chains to form a single "stalk."

Receptors for aggregated or insolubilized C1q were first recognized on lymphocytes (Dickler et al., 1972). Subsequent studies identified monocytes, some PMN, and B lymphocytes as the predominant C1q binding cell types in the peripheral blood (Tenner et al., 1981a). The molecular definition of the C1q receptor(s) has been difficult, which may be explained in part by different methods used to define different receptors on different cell types. Using C1q affinity, Reid and coworkers identified a 60 kDa protein (Malhotra et al., 1988, 1989) that is highly homologous, if not identical, to calreticulin (Malhotra et al., 1992; Stuart et al., 1996).

Initially using a purification procedure also based on C1q affinity, Ghebrehiwet and coworkers have described different forms of membrane-binding C1q molecules ranging from $1-2 \times 10^6$ M, (normal B lymphocytes) (Ghebrehiwet et al., 1982) to 70-80k M, (Raji cells) (Ghebrehiwet et al., 1984; Ghebrehiwet, 1988). The Raji cell C1qR had a domain with significant homology to $\alpha 5/\beta 1$ and the vitronectin receptor, $\alpha v/\beta 3$ integrin (Ghebrehiwet et al., 1992). More recently, this group has used antibodies against the 60k M, C1qR (Ghebrehiwet, 1988; Malhotra and Sim, 1989) and identified calreticulin on surface-labeled endothelial cells (Peerschke et al., 1993) and the T cell line MOLT4 (Chen et al., 1994).

Tenner and coworkers investigated the phagocytosis-enhancing and superoxide-enhancing effects of C1q on monocytes and granulocytes. They used a C1q affinity-purified fraction from U937 cells as an immunogen for making MAbs and then selected several MAbs that blocked C1q-enhanced phagocytosis by monocytes and found that the MAbs reacted with a 126k M, surface protein (Guan et al., 1991). Using degenerate probes based on amino acid sequence, they have identified a cDNA clone that directs expression of a protein with a predicted amino-terminal C-type lectin domain, five EGF-like repeats, a single transmembrane domain, and a short cytoplasmic tail (Nepomuceno et al., 1997). The 126k M, protein is expressed on both monocytes and neutrophils (Guan et al., 1991).

Results from our laboratory indicated that the regulation of C1q binding on human PMN was similar to the regulation of complement receptor type 1 (CR1) and type 3 (CR3) expression. Biotinylated C1q (C1q^{bio}) binding and CR1 and CR3 expression were simultaneously up-regulated by FMLP, and microtubule stabilization with taxol inhibited the up-regulation of C1q^{bio} binding and CR1 and CR3 expression (Jack et al., 1994). These findings suggested to us that the C1q receptor of PMN

^{††} Present Address: Department of Biostatistics, School of Public Health, University of Michigan, Ann Arbor, Michigan 48109.

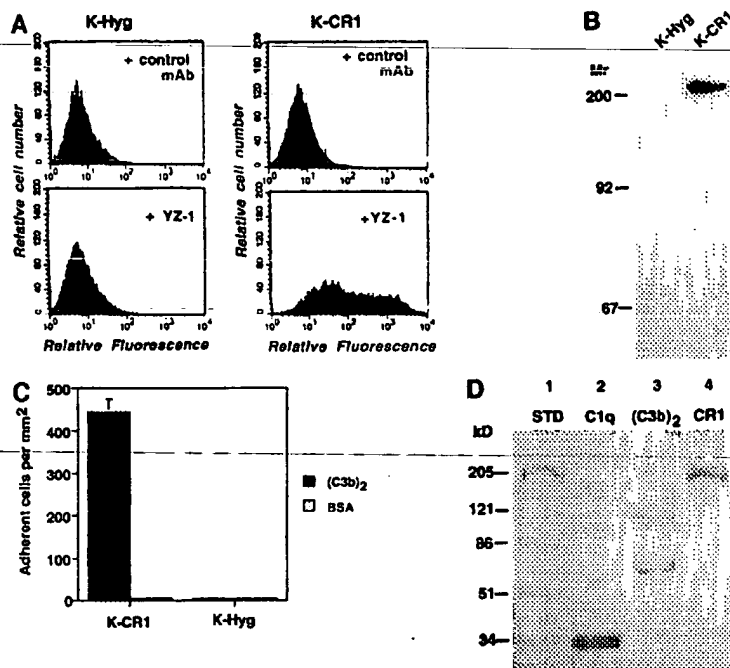


Figure 1. Analysis of CR1-Transfected K562 Cells

(A) FACS analysis of CR1 expression on transfected cells. Samples of cells transfected with the control plasmid pHyg (left panels) or the plasmid containing the cDNA encoding CR1 (right panels) were incubated with class-specific control MAb then FITC-goat anti-mouse IgG (upper panels), or anti-CR1 (YZ-1), followed by FITC-goat anti-mouse IgG (lower panels). The cells were subsequently fixed and analyzed by FACS. The K-Hyg cells (lower left) showed no positive staining for CR1, while the K-CR1 cells (lower right) displayed a broad histogram of positive staining. (B) Western blot analysis of NP-40 cell lysates of K-Hyg and K-CR1 cells using rabbit anti-CR1 and iodinated protein A as a probe. The K-CR1 cells have a unique 200k M_r band corresponding to intact CR1.

(C) Adherence of transfected K562 cells. K-CR1, but not K-Hyg, bound to C3b immobilized on plastic. Adherent cells were counted by microscopy.

(D) SDS-PAGE analysis under reducing conditions of purified proteins (4 μ g/lane) on a 4%–20% slab gel.

either was in the same intracellular storage vesicle as CR1 and CR3 or was identical to CR1 or CR3. The catalytic subunits of C1, C1r, and C1s, which bind C1q (Lepow et al., 1963), have complement short consensus repeat (SCR) motifs (Journet et al., 1986; Leytus et al., 1986; Tosi et al., 1987) and therefore share homology with CR1, which has 30 SCR in its extracellular domain (Klickstein et al., 1987). Thus, it was logical to assess if C1q could bind CR1.

In this paper, we provide evidence, from two different equilibrium binding assays and a kinetic binding assay, that C1q can indeed bind CR1. C1q has at least two binding sites for CR1, presumably involving some of its six identical stems, where C1r and C1s also bind (Siegel et al., 1983). These results indicate that CR1, the receptor normally responsible for binding immune complexes and mediating their transport to the liver and spleen for clearance, can bind to any of the complement fragments that mark immune complexes for clearance, namely C1q, C3b, and C4b.

Results

Characterization of Transfected Cells

The human erythroleukemia cell line K562 was transfected with the plasmid paABCD, which directs the expression of the F allotype of human CR1, together with the plasmid pBSHyg, which directs resistance to the antibiotic hygromycin (K-CR1), or with pBSHyg alone (K-Hyg). The K-CR1 and the control K-Hyg transfectants were characterized for CR1 expression using control MAb and the anti-CR1 MAb YZ-1. There was no YZ-1 staining of the K-Hyg cells, compared with a control MAb (MFC of FITC-second antibody alone = 9.25; MFC of anti-CR1 plus FITC-second antibody = 8.69) (Figure 1A, left panels). In the same analysis, K-CR1 demonstrated a heterogeneous pattern of positive staining compared with its control (Figure 1A, right panels). Because

the K-CR1 cells were selected for cell surface expression of CR1 by panning but the cells were not cloned, a heterogeneous expression of CR1 was expected. The recombinant CR1 molecule was shown to be intact by immunoprecipitation and Western blot analysis using polyclonal anti-CR1 (Yoon et al., 1985) and iodinated protein A as a probe (Figure 1B). The K-CR1 bound to C3b immobilized on plastic, while K-Hyg did not bind (Figure 1C). SDS-PAGE analysis of the purified proteins used in the experiments confirms their purity and expected molecular structures (Figure 1D).

Measurement of C1q Binding to Transfected Cells

Transfected cells were incubated at 37°C for 30 min with increasing amounts of biotinylated C1q (C1q^{bio}) in Ca⁺⁺- and Mg⁺⁺-free low ionic strength buffer, HBSS/2. Cells were subsequently incubated with FITC-avidin and analyzed by FACS. A plot of the mean fluorescent channel (MFC) versus C1q^{bio} input indicated specific binding of C1q^{bio} (Figure 2A). The binding was saturable and half maximal binding was seen at a C1q^{bio} concentration of 7.6×10^{-8} M. The C1q binding assay was repeated using ¹²⁵I-C1q. K-Hyg and K-CR1 cells were incubated with increasing amounts of ¹²⁵I-C1q for 45 min at room temperature. A dose-dependent increase in ¹²⁵I-C1q binding was observed (Figure 2B). However, it was not technically possible to reach saturation because the iodinated C1q aggregated at higher concentrations. A 50-fold excess of unlabeled C1q inhibited 82% (mean, n = 3) of 2.6 nM ¹²⁵I-C1q binding.

C1q Binding to CR1 Immobilized to Microtiter Wells

To circumvent the problem of background C1q binding to other cell surface sites, a recombinant, soluble construct of the entire extracellular domain of CR1 (rsCR1) was immobilized in microtiter wells. Preliminary titration of rsCR1 binding to the plate indicated that 0.5–0.8 μ g

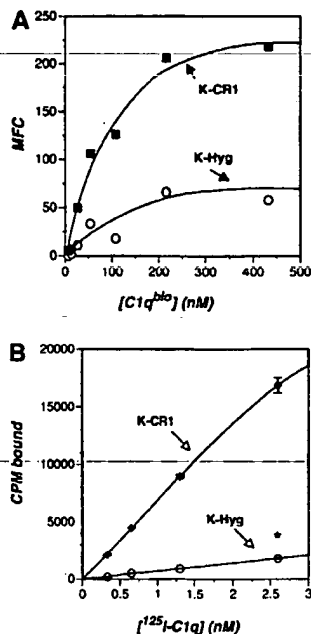


Figure 2. Labeled C1q Binding to Transfected Cells

(A) $C1q^{bio}$ Binding. After blocking nonspecific avidin and biotin binding sites, samples of K-Hyg and K-CR1 (5×10^6 cells) were reacted with increasing concentrations of $C1q^{bio}$ for 30 min at 37°C in low ionic strength buffer (HBSS/2; see Experimental Procedures for details). The cells were subsequently reacted with FITC-avidin, fixed in 1% paraformaldehyde, and analyzed by FACS. The mean fluorescent channel (MFC) of the single dominant peak was determined based on analyzing 20,000 cells. This experiment was repeated two other times with similar net positive binding of $C1q^{bio}$ to K-CR1 cells as compared with K-Hyg.

(B) ^{125}I -C1q binding. Transfected K-Hyg and K-CR1 cells, each at $5 \times 10^6/\text{ml}$, were incubated with increasing doses of ^{125}I -C1q (specific activity: $1.1 \times 10^6/\mu\text{g}$), diluted in low ionic strength buffer (HBSS/2). After a 45 min incubation at room temperature, three replicate 0.1 ml samples were removed from each reaction mixture, layered onto oil, and centrifuged to pellet the cells. The cell pellet containing 5×10^6 cells was cut off and counted. The means of triplicate values \pm SE are depicted. At the highest input of ^{125}I -C1q (2.6 nM), a 50-fold excess of unlabeled ligand inhibited specific binding by 82% (*). This experiment was repeated four times with similar results.

of rsCR1/well provided optimal C1q binding. C1q binding to cells was facilitated by low ionic strength, but C1q also aggregated at low ionic strength, which can make binding assays technically difficult. To identify an ionic strength closest to normal for the plate assay that would permit reproducible measurement of C1q binding to immobilized rsCR1, we tested the ability of $C1q^{bio}$ to bind under various ionic strength conditions. Adequate binding was measured in 0.1 M NaCl (Figure 3), thus subsequent plate binding studies were done in an equivalent low ionic strength buffer (" $0.67 \times \text{PBS-Tween}$ " = 67% PBS, 33% dHOH, 0.05% Tween-20, 5.9 mSi at 0°C).

The specific binding of ^{125}I -C1q to immobilized rsCR1 is shown in Figure 4A. Half maximal binding was observed at 0.5 nM of ^{125}I -C1q. The total binding observed in this assay was approximately 30-fold lower than that in the cell binding assay (Figure 2B) because of the higher ionic strength used in the plate assay. Binding of labeled C1q to immobilized rsCR1 was inhibited by

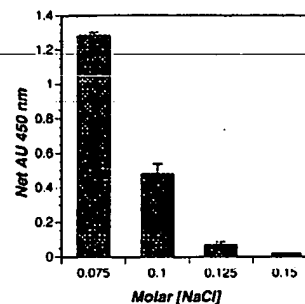


Figure 3. Effect of Ionic Strength on $C1q^{bio}$ Binding to Insolubilized rsCR1

Recombinant rsCR1 ($5 \mu\text{g}/\text{ml}$) was used to coat microtiter wells. After washing and blocking, $C1q^{bio}$ ($0.5 \mu\text{g}/\text{well}$), diluted in the respective ionic strength buffer, was added for a 30 min incubation at room temperature. The wells were washed $2 \times$ with the respective buffer. Results represent the mean of duplicate values, while the bars depict the range of values. This experiment was repeated with a similar inverse relationship between ionic strength and $C1q^{bio}$ binding.

native C1q (Figure 4B). A concentration of 0.4 nM unlabeled C1q inhibited ^{125}I -C1q binding by 50%. The collagen "tail" portion of C1q, which was obtained by pepsin digestion of C1q, also competed with ^{125}I -C1q for binding to immobilized rsCR1, which indicated that CR1 binds to the same collagen domain of the C1q molecule as does C1r and C1s (Siegel and Schumaker, 1983). In two experiments, 50% inhibition of ^{125}I -C1q binding was observed with about 10–15 nM of the collagen tails (data not shown). $(C3b)_2$ also inhibited ^{125}I -C1q for binding to CR1, but less efficiently than C1q (Figure 4C). Because $(C3b)_2$ is a well-described ligand for CR1 (Fearon, 1980), this affords additional specificity to the competition assays.

Kinetic Measurement of $C3b$ or C1q Binding to rsCR1

To demonstrate that C1q could bind CR1 under physiologic conditions and to avoid the deleterious effects of labeling procedures on the activity of C1q, surface plasmon resonance analysis of binding was performed using a BIAcore instrument. This technique measures in real time the association and dissociation of unlabeled ligand to an immobilized receptor, or vice versa, by changes in the adjacent refractive index (Cullen et al., 1987). In a typical experiment, rsCR1 was covalently coupled to a CM5 dextran chip and resulted in the net addition of 9052 resonance units (RU) to the chip.

To confirm that the coupled rsCR1 was functionally intact and to establish the validity of this method for measuring the binding of complement fragments to CR1, we assessed the ability of a known CR1 ligand, namely soluble $(C3b)_2$, to bind to the coupled rsCR1. Specific binding of $(C3b)_2$ to rsCR1 was observed using five different concentrations of $(C3b)_2$, ranging from 8.3 nM to 67 nM. Figure 5A illustrates the observed association and dissociation of $(C3b)_2$, which was used to derive the association and dissociation rate constants. The association phase of the interaction was modeled as simple bimolecular binding, because the $(C3b)_2$ ligand was employed at a concentration 20- to 60-fold below

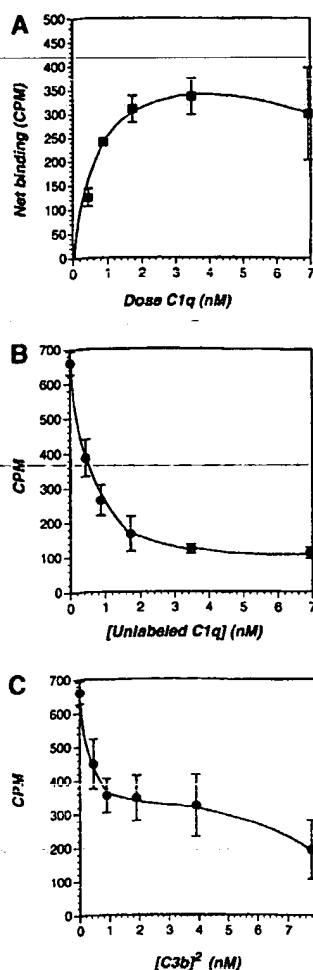


Figure 4. Binding of ¹²⁵I-C1q to Immobilized rsCR1 and Competition by Unlabeled Ligands

Recombinant sCR1 (8 μg/ml) was immobilized in microtiter wells, and binding was performed in 0.67 × PBS-Tween buffer in the absence of added Ca⁺⁺ and Mg⁺⁺, as described in the Experimental Procedures.

(A) Direct ¹²⁵I-C1q binding to plated rsCR1. Results are the means ± SE, n = 4. This experiment was repeated with similar results.

(B) Competition of ¹²⁵I-C1q binding by native C1q. ¹²⁵I-C1q (0.87 nM) binding to immobilized rsCR1 was measured in the presence of increasing concentrations of unlabeled C1q in 0.1 M buffer. Means ± SE, n = 3 are plotted. This experiment was repeated with similar results. Unlabeled C1q at 0.4 nM inhibited ¹²⁵I-C1q binding by 50%.

(C) Competition of ¹²⁵I-C1q binding by (C3b)₂. ¹²⁵I-C1q (0.87 nM) was added to wells containing immobilized rsCR1 in the presence of increasing amounts of (C3b)₂. Results are the means ± SE, n = 4.

the value of the reported equilibrium dissociation constant (K_{eq}) of monomeric C3b for CR1 (Amaout et al., 1981). Further, the sequential binding of two identical binding sites would not significantly change the observed association kinetics (Gertler et al., 1996). At the time the dissociation phase begins, the (C3b)₂ may be bound monovalently or divalently; thus, a curve-fitting model assuming parallel dissociation of two distinct complexes was employed. The association and dissociation models closely fit the experimental data (not

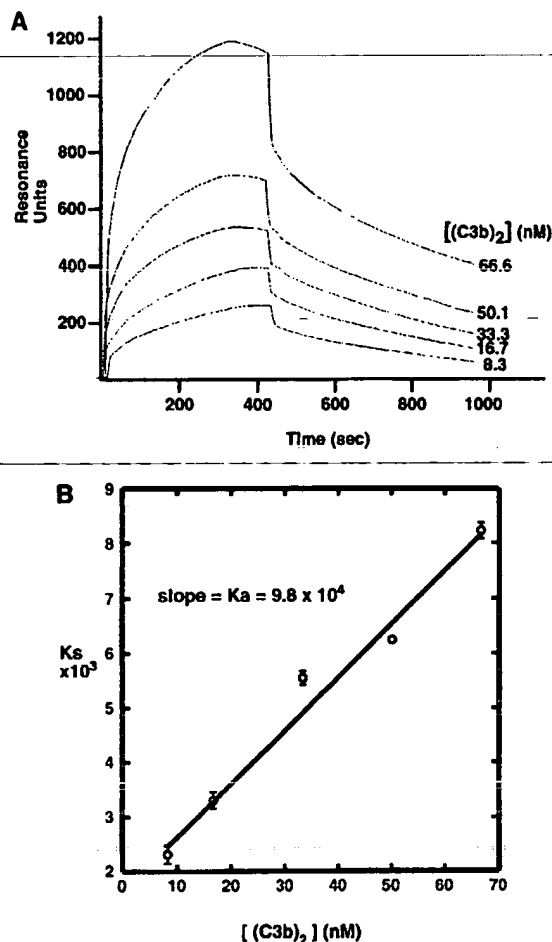


Figure 5. Kinetic Binding Data: C3b Dimers Bind to CR1, Documenting That Immobilized rsCR1 Is Functional

(A) Plot of resonance units (RU) versus time illustrating the observed association and dissociation of (C3b)₂. The association was monitored from 0 to 300 sec, and the dissociation occurred during the interval from 450 to 600 sec.

(B) Data from (A) were used to plot K_s versus [(C3b)₂]. The slope of the least squares regression line ($r^2 = 0.977$) corresponds to the apparent association rate constant.

shown). Table 1 contains the observed rate constants, k_a and k_d , and the calculated equilibrium dissociation constant, K_{eq} , which corresponds to the ligand concentration at which half of the receptors are occupied. The K_{eq} for (C3b)₂ binding to CR1 was calculated for the five (C3b)₂ concentrations used and averaged 27.6 nM, with a range from 17.9 nM to 40.6 nM. An alternative analysis of the association kinetics, which does not assume a known k_d , yielded a k_a of $9.79 \times 10^4 \text{ mol}^{-1} \times \text{sec}^{-1}$, an apparent k_d of $1.7 \times 10^{-3} \text{ sec}^{-1}$, and a calculated K_{eq} of 17.4 nM (Figure 5B). Using the more reliable average k_{eq} determined from the dissociation kinetics, $2.078 \times 10^{-3} \text{ sec}^{-1}$ (Table 1), the calculated K_{eq} for (C3b)₂ binding to CR1 is 21.2 nM, in good agreement with the first method. These values are not significantly different from the previous determination of 9.5 nM for a similarly prepared (C3b)₂ (Amaout et al., 1981), thus validating our methods.

Table 1. Kinetic Data for (C3b)₂ Binding to Immobilized CR1

[C3b] ₂ nM	k _a (SE) × 10 ⁻⁴ mol ⁻¹ × sec ⁻¹	k _{off} (SE) × 10 ³ sec ⁻¹	k _{on} (SE) × 10 ³ sec ⁻¹	K _{eq1} (SE) μM	K _{eq2} (SE) nM
8.3	5.5 (1.8)	65.8 (15.8)	1.9 (0.9)	1.2 (0.49)	34 (19.8)
16.7	6.0 (0.9)	276 (11.1)	2.4 (0.04)	4.6 (0.71)	40 (6.0)
33.3	10.5 (0.3)	261 (14.2)	2.2 (0.05)	2.5 (0.15)	21 (0.77)
50.1	8.3 (0.2)	346 (46.6)	2.1 (0.05)	4.2 (0.57)	25 (0.85)
66.7	9.9 (0.2)	110 (17.7)	1.8 (0.4)	1.1 (0.18)	18 (4.0)
				$\bar{x} = 2.7 \mu\text{M} (1.06)$	$\bar{x} = 27.6 \text{ nM} (21.1)$

In a similar analysis using five different concentrations of unlabeled C1q ranging from 6.5 to 52.2 nM, binding to CR1 was evident in normal ionic strength buffer (Figure 6A). No C1q binding was observed to a parallel channel that was not derivatized, or to a channel that was derivatized with C1q, data that demonstrated the specificity of C1q binding to rsCR1 in this assay. Using the same kinetic models as used for (C3b)₂ binding, a good fit to the experimental data was obtained. Analysis of the dissociation data for the more rapidly dissociating complex yielded an apparent k_d that ranged from 0.019 to 0.033 sec⁻¹ among the five concentrations analyzed. The more slowly dissociating complex had an apparent k_d that ranged from 0.84 × 10⁻³ to 2.09 × 10⁻³ sec⁻¹.

Using the latter dissociation constants in a simple association model, the apparent k_a ranged from 2.86 × 10⁵ to 4.34 × 10⁵ mol⁻¹ × sec⁻¹ (Table 2). The calculated K_{eq} for the slowly dissociating C1q-CR1 complex averaged 3.9 nM, with a range from 2.27 nM to 5.17 nM. An alternative analysis of the association kinetics, which does not assume a known k_d, yielded a k_a of 4.34 × 10⁵ mol⁻¹ × sec⁻¹, an apparent k_d of 1.95 × 10⁻³ sec⁻¹, and a calculated K_{eq} of 4.49 nM (Figure 6B). Using the more reliable average k_d determined from the dissociation kinetics, 1.49 × 10⁻³ sec⁻¹, the calculated K_{eq} for C1q binding to CR1 was 3.43 nM, in good agreement with the first method. The most likely interpretation of these data is that the slowly dissociating complex interacted with CR1 via more of the six identical stems of C1q than did the rapidly dissociating complex. Importantly, C1q has a higher apparent affinity for CR1, under normal ionic conditions, than does (C3b)₂.

When C1q was directly immobilized to the chip by NHS/EDC chemistry, we were unable to detect rsCR1 binding in isotonic buffer (data not shown). In retrospect, this was probably because the C1q was denatured during the coupling reaction, which has been observed with other proteins (Gertler et al., 1996). However, when C1q^{bio} was immobilized to a streptavidin chip, it was possible to detect CR1 binding, but only at relatively high concentrations of rsCR1 (435 nM to 3480 nM). The very rapid association and dissociation of rsCR1 to the immobilized C1q^{bio} precluded an accurate determination of the rate constants by the BIAcore technique (BIA Evaluation and BIA Simulation, Pharmacia). The most likely explanation for why C1q bound at high apparent affinity to immobilized CR1, while soluble CR1 bound poorly to immobilized C1q, is that C1q has a higher valency for CR1 than CR1 has for C1q. For example, C1q with its six identical stems may have six binding sites for CR1, while CR1 apparently has fewer sites for binding to C1q.

Localization of a Binding Site in LHR-D for C1q

Using deletion mutations that previously had been employed to define the binding sites for C4b and C3b (Klickstein et al., 1988), we found that ¹²⁵I-C1q tails consistently bound to a fragment of rCR1 containing the nine SCR closest to the membrane. This region includes the fourth LHR, LHR-D (SCR 22-28) (Figure 7). Because all of the constructs studied contained all or part of LHR-D, we could not exclude additional binding sites in LHR-A, -B, or -C. Computer-aided sequence alignment (Wisconsin Package, Version 8, Sept. 1994, Genetics Computer Group, 575 Science Drive, Madison, WI 53711) revealed that the SCR in C1r and C1s are most

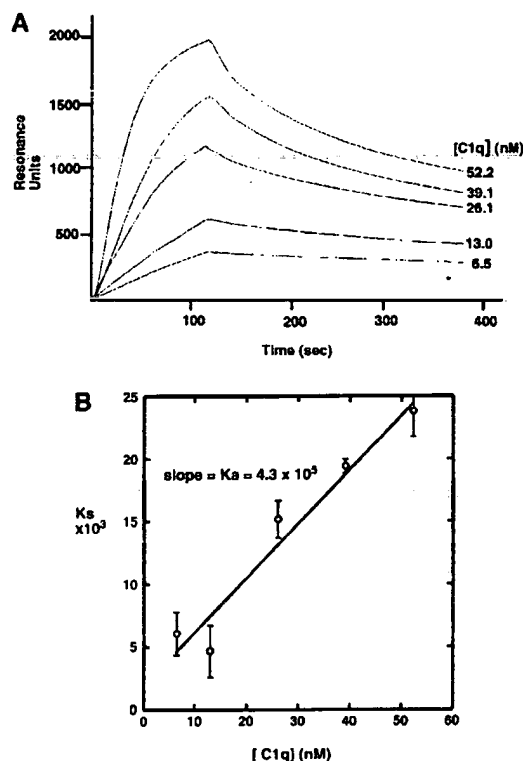


Figure 6. Kinetic Binding Data: C1q Binds to Immobilized rsCR1

(A) Plot of resonance units (RU) versus time illustrating the observed association and dissociation of C1q in normal saline. The association was monitored from 0 to 120 sec, and the dissociation occurred during the interval from 120 to 250 sec.

(B) Data from (A) were used to plot K_s versus [C1q]. The slope of the least squares regression line (r² = 0.947) corresponds to the apparent association constant, determined without assuming knowledge of the dissociation rate constant.

Table 2. Kinetic Data for C1q Binding to Immobilized CR1

[C1q] nM	k_a (SE) $\times 10^5$ $\text{mol}^{-1} \times \text{sec}^{-1}$	k_{d1} (SE) $\times 10^3 \text{ sec}^{-1}$	k_{d2} (SE) $\times 10^3 \text{ sec}^{-1}$	K_{d1} (SE) nM	K_{d2} (SE) nM
6.5	3.7 (2.63)	23.6 (4.8)	0.84 (0.03)	64 (47.3)	2.3 (1.64)
13.0	2.7 (1.08)	22.3 (3.4)	1.24 (0.04)	83 (35.5)	4.3 (1.73)
26.0	4.3 (0.57)	23.7 (1.5)	1.54 (0.03)	55 (8.1)	3.6 (0.48)
39.0	4.3 (0.13)	19.1 (1.1)	1.74 (0.06)	44 (2.9)	4.0 (0.18)
52.0	4.0 (0.285)	32.7 (1.9)	2.09 (0.04)	82 (7.5)	5.2 (0.38)
				$\bar{x} = 59 \text{ nM (60.2)}$	$\bar{x} = 3.9 \text{ nM (2.47)}$

homologous to SCR of LHR-D, which is consistent with LHR-D containing a CR1 binding domain for C1q.

Discussion

CR1 (CD35) is a single-chain, integral membrane glycoprotein that has been recognized as the major cellular receptor for C4b and C3b (Fearon, 1980). The extracellular domain of the protein is comprised of SCR, which are characteristic of C3/C4 binding proteins (reviewed in Hourcade et al., 1989). The rsCR1 used in these studies is truncated after SCR 30. Groups of 7 SCRs in CR1 are further organized into 4 long homologous repeats (LHR-A, -B, -C, and -D) (Klickstein et al., 1987). LHR-A contains a C4b binding site, and LHR-B and LHR-C each contain a binding site for C3b (Klickstein et al., 1988; Krych et al., 1991), which also binds C4b, but at a lower affinity (Reilly et al., 1994). We have demonstrated C1q binding to CR1 by three different means: (1) equilibrium binding of labeled C1q to CR1-transfected K562 cells (Figures 2A and 2B); (2) equilibrium binding of labeled C1q (Figures 3 and 4) and labeled C1q tails (Figure 7) to plated rsCR1; and (3) real-time binding of unlabeled C1q to immobilized rsCR1 in normal saline (Figures 6A and 6B). Although there are likely other C1q binding

sites on CR1, we consistently detected good binding of ^{125}I -collagen tails to a construct containing LHR-D (SCR-22-28) and the last two SCR of the extracellular domain, SCR 29 and 30. Interestingly, the region of CR1 most homologous to the SCR of C1r and C1s is LHR-D. Until now, there has been no binding function assigned to this region.

The (C3b)₂ partially inhibited binding of C1q to immobilized CR1. One possible explanation for the partial inhibition is that the binding sites for the two ligands differ, but are close enough that (C3b)₂ sterically hinders C1q binding. Both (C3b)₂ and C1q are large molecules, ~360,000 and 462,000 daltons, respectively. A second possibility is that there is more than one binding site for C1q that accounts for the partial inhibition of C1q binding by (C3b)₂. This would be consistent with our suspicion that there is an additional binding site for C1q outside of LHR-D (data not shown). Thirdly, the binding of (C3b)₂ may affect the tertiary structure of CR1 in a way that inhibits C1q binding. These three possibilities are not mutually exclusive.

Derivatization of ligands always risks introducing artifacts, and C1q is especially sensitive to manipulation. It was reported recently that C1q^{bio}, along with other biotinylated proteins, failed to show any specific binding to cells (Storm et al., 1996). We have found that in the case of C1q, its specific binding to PMN (Jack et al., 1994), CR1-transfected cells, and to plated CR1, and its residual hemolytic activity are adversely affected by high levels of biotinylation. However, with low levels of biotinylation, and also helped by the use of avidin/biotin blocking reagents (Vector Labs), C1q^{bio} demonstrates specific binding to K-CR1 (Figure 2A). During the course of these experiments, we found that the hemolytic function of C1q was more sensitive to biotinylation than the binding site for CR1. Biotinylation with NHS-biotin at 0.6 $\mu\text{g/ml}$ and 30 $\mu\text{g/ml}$ yielded two preparations of C1q^{bio} with near normal binding to CR1, but with 23% and 90% losses in hemolytic activity, respectively. Radiolabeling C1q has also been shown to increase the dissociation constant of C1r and C1s from C1q (Tseng et al., 1997). In our plate binding assay performed at 2/3 normal ionic strength (5.9 mSi), the 1/2 maximal binding of C1q (Figure 4A) and 1/2 maximal inhibition by unlabeled C1q (Figure 4B) were about 0.5 nM. Surface plasmon resonance analysis, on the other hand, allowed the direct measurement of binding by nonderivatized C1q in normal ionic strength buffer. The affinity values derived from the two methods are internally consistent, considering that C1q would be expected to have a lower binding affinity in the plasmon resonance analysis because of the adverse effect of a higher salt concentration on

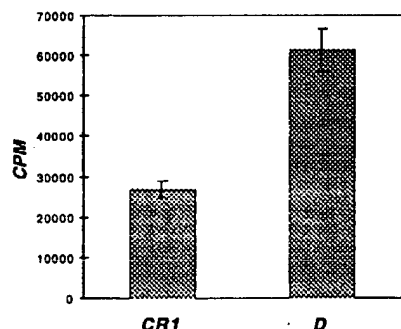


Figure 7. Binding of ^{125}I -C1q Collagen Tails to Immobilized Constructs of CR1

Microtiter wells were coated with anti-LHR-D (Mab 6B1, T Cell Sciences) at 3 $\mu\text{g/ml}$ and blocked with milk. NP-40 lysates of equal numbers of control, and transfected CHO cells were added to allow the MAb to capture the constructs. Lysates of nontransfected CHO cells were added to some wells to allow determination of nonspecific binding. After washing wells, ^{125}I -C1q collagen tails (10 nM) were added for binding in 0.67 \times PBS-Tween binding buffer (5.9 mSi). Results (mean \pm SE, $n = 4$) are the specific binding, which has been normalized to equal molar amounts of constructs (see Experimental Procedures). This experiment was repeated using binding buffers of 3.75, 4.44, and 5.9 mSi with similar results.

binding to rsCR1 (Figure 3). It is interesting to note that both C1r₂C1s₂ binding and CR1 binding to C1q occur in the collagen domain (Figure 7) and both binding reactions are strongly inhibited by salt (Siegel and Schumaker, 1983; Tseng et al., 1997). CR1, C1r, and C1s all contain SCR units; therefore, we propose that there is a common binding site on the collagen stems of C1q for SCR-containing proteins. This hypothesis is supported by the observation that when C1r₂C1s₂ is bound to C1q, the C1q is unable to bind to C1qR-bearing cells (Tenner et al., 1980).

An intriguing question is why CR1 was not identified as a C1q receptor previously. While this may have to do with the low density of CR1 expression on resting cells (Arnaout et al., 1981; Fearon et al., 1983), the most likely explanation is that insolubilized C1q is a poor affinity ligand for CR1. If the K_{d0} for CR1 binding to immobilized C1q were in the range of 0.3–3 μ M, where binding was observed to immobilized C1q^{bio} in the BIAcore analysis (data not shown), then the CR1 concentration in cell lysates would be far too low to detect specific binding. The presence of detergent used to solubilize cell membranes might further impair binding. The probable explanation for why CR1 binds poorly to immobilized C1q, while C1q binds at high apparent affinity to immobilized CR1, is that C1q has a higher valency for CR1 than CR1 has for C1q. For example, C1q with its six identical stems may have six binding sites for CR1, while CR1 has fewer sites for binding to C1q. Consistent with this hypothesis, CR1 on the surface of cells is highly clustered (Petty et al., 1980; Edberg et al., 1987; Paccaud et al., 1988, 1990; Chevalier et al., 1989), which would allow one molecule of C1q to interact with multiple CR1 molecules.

The role of CR1 with respect to other molecules proposed as C1q receptor will have to be defined. Calreticulin has been proposed as a C1q receptor, but it is primarily found in the ER, where it acts as a Ca⁺⁺-binding protein and as a chaperonin for nascent proteins. Perhaps as a consequence of this chaperone function, calreticulin is also found in the granule fraction of some cells (reviewed in Bleackley et al., 1995) and might be expressed on cells as a consequence of degranulation and the adsorption to plasma membrane (Eggleton et al., 1994). It is unclear how such adsorbed calreticulin could act as a receptor. Recent work indicates that calreticulin preferentially binds to the globular domain, as opposed to the collagen domain of C1q (Kishore et al., 1997). These data have not been reconciled with the putative role of calreticulin as the C1q receptor for the collagen domain. Antibodies to a recently cloned 126k M_r transmembrane protein block the C1q-stimulated phagocytosis by monocytes (Guan et al., 1991; Nepomuceno et al., 1997). No binding of C1q has been demonstrated to this novel 126k M_r protein; thus, it may participate as an element of a larger C1q receptor complex. If the amino-terminal C-type lectin domain of this transmembrane protein were responsible for binding C1q, one would expect that C1q binding to cells would be Ca⁺⁺-dependent, which was not observed (Jack et al., 1994).

Although C1q has many biological effects presumably mediated through cell surface receptors, (reviewed in Ghebrehiwet et al., 1993; Tenner, 1993), there is little

known about how CR1 might mediate transmembrane signaling. CR1 participation in the cellular responses to C1q might require that other molecules be recruited. There is precedence for CR1 associating with other molecules: CR1 is known to associate with CD21 in the membrane of B cells (Tuveson et al., 1991; Matsumoto et al., 1993), and when CR1 is cross-linked, it forms attachments to the cytoskeleton (Jack et al., 1986; Brown, 1989). Finally, CR1 may not be the only C1q receptor, since C1q reportedly does bind and activate some cells, e.g., endothelial cells (Zhang et al., 1986; Lozada et al., 1995), which do not express detectable CR1 (Shaw et al., 1995).

There are important biological implications for C1q/CR1 binding. CR1 on erythrocytes is critical for the transport of immune complexes and their subsequent clearance. Thus, CR1 in humans may be solely responsible for clearing immune complexes opsonized by complement, whether C1q and/or C3b/C4b. That C1q might participate in the clearance of immune complexes may explain several clinical observations. It is well recognized that deficiency of an early component of the classical pathway predisposes to autoimmune diseases, typically SLE (Lachmann, 1984; Atkinson, 1986). Only about half of the patients with homozygous C2 deficiency have lupus-like disease characterized primarily by cutaneous manifestations, and vital organ involvement is uncommon (Colten et al., 1992). In contrast, C1q deficiency is almost invariably associated with SLE (31/33 patients) (Petty et al., 1997), and the disease is very severe, often including central nervous system pathology, glomerulonephritis, and the presence of autoantibodies (Bowness et al., 1994). The clinical manifestations of homozygous C4 deficiency are intermediate in severity between those associated with C2 and C1q deficiency (Colten and Rosen, 1992). Deficiency of C2 would result in defective opsonization by C3 only, while deficiency of C4 would result in defective opsonization by both C4 and C3. However, immune complexes might still be effectively opsonized by C1q. In C1q deficiency, however, neither C1q, C3b, nor C4b would effectively bind to immune complexes, and the complexes might then be deposited in vital organs such as the kidney rather than the normal targeting to liver and spleen for clearance (reviewed in Hebert, 1991). An alternate hypothesis places the critical role of C1q in preventing autoimmune disease before the formation of immune complexes. In a recently published study, C1q has been shown to directly bind to the blebs of apoptotic keratinocytes (Korb et al., 1997). Because these blebs are enriched in nuclear antigens recognized by autoantibodies in SLE, it is hypothesized that C1q is normally involved in the clearance/tolerance of these antigens.

The mechanism underlying the severe autoimmune disease associated with C1q deficiency may be due to the inability to activate the classical pathway or the inability to recruit C1q receptor-bearing cells. Now that CR1 has been identified as a C1q receptor, it will be possible to address these alternatives.

Experimental Procedures

Buffers/Reagents

PBS used in microtiter well assays: 0.15 M NaCl, 0.05 M sodium/potassium phosphate (pH 7.4). PBS used in plasmon resonance

assays: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 (pH 7.3). The C1q binding buffer used with transfected cells was "HBSS/2" = 1 vol HBSS without $\text{Ca}^{++}/\text{Mg}^{++}$:1 vol 5% glucose-0.2% gelatin solution). The C1q binding buffer used in the microtiter plate assay was 0.67 \times PBS-Tween = 67% PBS, 33% dHOH, 0.05% Tween-20, 5.9 mSi at 0°C. The following reagents were purchased as noted: 1,3 diaminopropane (Sigma Chemical, St. Louis, MO), dinonyl phthalate (Arcos, Geel, Belgium), dibutyl phthalate (Sigma). Recombinant soluble human CR1 (rsCR1) was kindly provided by Drs. Una Ryan and Henry Marsh of T-Cell Sciences, Needham, MA.

Complement C1q and Collagen Tails of C1q

C1q for biotinylation (as used in Figure 2A) was isolated from human serum by a procedure using BioRex 70, as originally described (Tenner et al., 1981b) and modified (Jack et al., 1994). Each batch of C1q^{iso} was tested for functional activity (Tenner et al., 1981b). Because of the propensity of the native C1q made by the BioRex method to aggregate, an alternative isolation method for C1q, utilizing fractional euglobulin precipitation and gel permeation chromatography, was devised to prepare C1q for radiolabeling. In brief, 30 ml of fresh serum, 5 mM EDTA was dialyzed against a euglobulin precipitation buffer (20 mM morpholine ethane sulfonic acid, 5 mM benzamidine, 0.5 mM EDTA [pH 6.5]) in tubing for 16 hours at 4°C. The precipitate was collected by centrifugation (8000 \times g, 10 min), washed twice in 5 mM propanediamine, 0.05 mM EDTA (pH 8.8) (PDE buffer) (Liberti et al., 1981), then dissolved in 1.4 ml of PDE buffer supplemented with 300 mM NaCl (PDE/NaCl), 0.5 mM PMSF. The mixture was centrifuged at 10,000 \times g for 5 min to remove undissolved precipitate. The supernatant containing C1q was aliquoted (0.45 ml) to each of three microfuge tubes. Cold water (1 ml) was added to each tube, and after 10 min at 4°C, the resulting precipitates were collected by centrifugation (10,000 \times g, 5 min). The precipitates in each tube were dissolved in 75 μ l of PDE/NaCl buffer, and then 85 μ l of 2 \times PBS was added. This material was recentrifuged (15,000 \times g, 5 min), and 150 μ l was applied to a TSK G4000SW_{XL} column (Supelco, Bellefonte, PA) equilibrated in 2 \times PBS, 0.5 mM EDTA at a flow rate of 0.5 ml per min. C1q eluted as a peak with a retention time of 18.6 min consistent with a MW of 462 kDa, and the peak was collected by hand. Analysis of the C1q revealed the distinct a, b, and c chains by SDS-PAGE with silver staining. The specific activity of the C1q was 400 hemolytic units (Z) per μ g of protein using the assay based on the BioRex drop through fraction of human serum (Tenner et al., 1981b). Protein was assayed by the micro BCA (bicinchoninic acid method; Pierce Chemicals, Rockville, IL), and C1q concentrations were calculated from a BSA standard curve.

The collagen domain of C1q was prepared from a pepsin digest of C1q (Siegel and Schumaker, 1983) and purified by HPLC gel permeation chromatography using the same TSK G4000SW_{XL}, vide supra. Protein was quantified with the BCA assay, using BSA as a standard.

Biotinylation of C1q

In a typical biotinylation reaction, 840 μ g of C1q was reacted with 4.3 μ g of NHS-biotin (Pierce Chemicals, Rockville, IL) in 1 ml of PBS for 30 min at room temperature with intermittent agitation. The reaction was stopped by the addition of concentrated ethanolamine to give a final concentration 0.1 M. The reaction mixture containing C1q^{bio} was subsequently dialyzed against 120 mM KCl, 10 mM Tris/HCl (pH 7.4) and the protein assayed. We were not able to determine the final biotin:C1q ratio using the reagents provided with the biotinylation kit, but it was necessary to biotinylate "lightly" to avoid aggregation. Each batch of C1q^{bio} was tested for functional activity (Tenner et al., 1981b).

Radiolabeled C1q and C1q Collagen Tails

Glucose oxidase (Sigma) and lactoperoxidase (Sigma) were separately coupled to beads of cross-linked bis-acrylamide/azlactone copolymer beads (3M Emphaze; Pierce Chemicals, Rockville, IL) according to the manufacturer's instructions. For both enzymes, the coupling ratio was 1 mg of protein per hydrated equivalent of 24 mg of dried beads. The optimal ratio (1/4) of coupled glucose oxidase to coupled lactoperoxidase was determined in a preliminary

experiment by combining the beads in different ratios and measuring the resultant enzymatic activity using 0.1% D-glucose in tetramethylbenzidine solution (Kirkegaard and Perry, Gaithersburg, MD) as the substrates. The development of blue color was followed spectrophotometrically.

At the time of radiolabeling, glucose oxidase beads (3 μ l of a slurry) and lactoperoxidase beads (12 μ l of a slurry) were washed into PBS. Sodium-¹²⁵Iodine (3 μ l, carrier free, 100 mCi/ml, New England Nuclear, Boston, MA) was added to the bead pellet, followed by 70 μ l of C1q (400 μ g/ml in 2 \times PBS), 70 μ l of dHOH, and 10 μ l of glucose solution (100 μ g/ml PBS). The reaction proceeded for 20 min at room temperature with intermittent shaking. The reaction supernatant was applied to a PD-10 gel filtration column (Pharmacia), which had been equilibrated in PBS, 0.1% gelatin. The radio-labeled C1q was pooled and characterized. 98% of CPM of the ¹²⁵I-C1q were precipitable with 10% TCA. ¹²⁵I-C1q was quantified by a sandwich ELISA using anti-C1q MAb (Quidel, San Diego, CA) as the capture antibody and goat anti-human C1q (IncStar, Stillwater, MD) as the indicator antibody. The reaction was developed with horseradish peroxidase-conjugated rabbit anti-goat IgG (IncStar) and tetramethylbenzidine substrate. The color reaction was stopped by the addition of H_2PO_4 , and the OD₄₅₀ was quantified using an ELISA plate reader (Molecular Devices, Menlo Park, CA). C1q of a known protein concentration was used as a standard. Multiple lots of C1q were iodinated with specific activity ranging from 5×10^5 – 1×10^6 CPM per μ g of C1q as quantified by ELISA, and the functional activity was 400 hemolytic units (Z) per μ g.

Collagen tails of C1q were radioiodinated using IodoGen (Pierce Chemicals, Rockville, IL) to a specific activity of 2.4×10^5 cpm/ μ g of protein.

Preparation of (C3b)₂

C3 purified from fresh human plasma by standard methods (Hammer, 1981, #248) was treated with trypsin to produce C3b (Fearon, 1983, #638). The trypsin was inactivated by addition of diisopropyl fluorophosphate and the C3b purified by chromatography on Sepharose CL-2B in PBS. The fractions containing C3b were pooled, concentrated to 1.4 mg/ml (Centriprep, Amicon, Beverly, MA), and stored at 4°C for 3 weeks to allow formation of dimers via oxidation of the free sulfhydryl group, as occurs in stored C3 (Amaout et al., 1981). (C3b)₂ was separated from monomeric C3b by gel filtration on Sepharose CL-2B in PBS (Pharmacia LKB Biotechnology, Piscataway, NJ), and the expected M_r was confirmed by SDS-PAGE. Peak fractions were pooled, aliquoted, and stored at -80°C.

Preparation of K562 Cells with Cell Surface CR1

The plasmid pBSHyg, which directs the expression of hygromycin resistance, was prepared by ligation of the 2.0 kb HindIII-NruI fragment from REP3 (Groger et al., 1989) into the HindIII-HincII sites of Bluescript KS(-) (Stratagene, La Jolla, CA). The plasmid paABCD directs the expression of the F allotype of human CR1 (Klickstein et al., 1988). K562 cells were electroporated (250 V, 960 μ F) with 200 ng of pBSHyg linearized with XmnI with or without 20 μ g of paABCD linearized with SfiI. Transfectants were selected by culture in RPMI with 20% FCS supplemented with hygromycin at 200 μ g/ml for 2 weeks, then transferred to RPMI with 10% FCS. K562 cells transfected with pBSHyg alone were termed K-Hyg, and those transfected with paABCD were termed K-CR1. The K-CR1 cells were immunopanned (Wysocki et al., 1978) on immobilized anti-CR1 MAb (YZ-1) (Changelian et al., 1985) to select a uniformly positive population of cells. The CR1 expression on the control K-Hyg and K-CR1 transfectants was assessed by YZ-1 and FACs analysis, vide infra. Once during the period of experimentation, the K-CR1 transfectants were reselected by panning using YZ-1 to enhance CR1 expression. Western blotting of transfected cells to detect CR1 was performed as described (Yoon and Fearon, 1985). For the adhesion assay, (C3b)₂ was adsorbed to plastic wells in 50 mM Tris buffer (pH 9), and the plate was blocked with 1% BSA. K562 transfectants were added and allowed to bind for 30 min at room temperature. After gently washing the plates, the adherent cells were enumerated using light microscopy.

FACS Analysis

A FACStar (Becton Dickinson, San Jose, CA) was used, and 10⁴ cells were analyzed for each variable. Transfected cells were assessed for

CR1 expression using the murine MAb YZ-1 and FITC-goat anti-mouse IgG (Tago-Biosource, Camarillo, CA).

¹²⁵I-C1q Binding to Transfected Cells

Binding was performed in a polypropylene microfuge tube using "HBSS/2" (HBSS without Ca⁺⁺ and Mg⁺⁺), which was diluted with an equal volume of 5% glucose-0.2% gelatin solution. The total reaction volume of 0.33 or 0.44 ml contained 5 × 10⁶ cells/ml and increasing amounts of ¹²⁵I-C1q (specific activity: 1.1 × 10⁶ cpm/μg). The binding reaction proceeded at room temperature for 45 min with regular agitation. Aliquots (0.1 ml) of the reaction mixture were removed and layered onto 300 μl of an oil mixture (85% dibutyl phthalate, 15% dinonyl phthalate) in microfuge tubes (0.4 ml of polyethylene, #1404-1000, USA/Scientific Plastics, Ocala, FL). The tubes were spun for 2 min at 9000 × g (Microfuge B, Beckman Instruments, Fullerton, CA), and the tips containing the cell pellets were cut off and counted in a gamma counter.

C1q^{ab} Binding to Transfected Cells

Cells were first reacted with avidin/biotin blocking reagents following the manufacturer's instructions (Vector Laboratories, Burlingame, CA) for 10 min at room temperature. Subsequently, 5 × 10⁶ cells were aliquoted to tubes and HBSS/2 buffer ± dilutions of C1q^{ab} were added (final volume, 135 μl) and incubated for 30 min at 37°C. After appropriate washes, FITC-avidin (Vector)-diluted 1/250 was added for 25 min at room temperature. All later wash and incubation steps were performed in the same HBSS/2 buffer. After a wash, the cells were fixed in 1% paraformaldehyde/PBS and analyzed by FACS.

C1q Binding to Insolubilized rsCR1

Microtiter wells (Immulon 1 Removawell strips, Dynatech Labs, Alexandria, VA) were treated with 0.1 ml of rsCR1 (5 or 8 μg/ml, both saturating concentrations) diluted in coating buffer (0.01 M Na₂CO₃, 0.04 M NaHCO₃, pH 9.6) for 2 hours at 37°C or overnight at 4°C. For the C1q^{ab} binding studies, wells were blocked with SuperBlock (Pierce Chemicals, Rockville, IL), per the manufacturer's instructions. For binding studies using ¹²⁵I-C1q or ¹²⁵I-collagen tails, blocking was done for 2 hr at 37°C with 3% nonfat dried milk (BioRad, Hercules, CA), 0.5% Tween-20 (Baker Chemical, Phillipsburg, NJ) in PBS.

Varying amounts of ¹²⁵I-C1q in 0.67 × PBS-Tween binding buffer were incubated in the rsCR1-coated wells for 45 min at room temperature in the presence or absence of a competitive ligand. Total reaction volume was always 100 μl. After the binding incubation, the wells were emptied by aspiration and then the plate was turned upside down and vigorously slapped five times against layers of filter paper. Subsequently, the wells were separated and individually counted in a gamma counter.

C1q Binding to Insolubilized Deletional Mutants of CR1

Microtiter wells were coated with MAb anti-LHR-D (3 μg/ml) (clone 6B1, gift of Dr. Henry Marsh, T-Cell Sciences, Needham, MA) as described for CR1 above. Clone 6B1 binds an epitope within the region of SCR 26-30 (data not shown). Wells were blocked with dried milk as described above. NP-40 lysates of control CHO cells, or CHO cells that were transfected with either the full-length CR1 (piABCD) or LHR-D (piD) (Klickstein et al., 1988) were added to the wells for 2 hr at room temperature to allow the immobilized anti-LHR-D to capture the recombinant CR1 antigen. After washing the wells three times with binding buffer (vide supra), ¹²⁵I-collagen tails (10 nM) in binding buffer were added for 45 min at room temperature. After two washes in binding buffer, the wells were separated and individually counted in a gamma counter. To normalize the binding data, compensation was made for the molar amount of LHR-D antigen in each lysate. A two-site capture radioimmunoassay was performed that used a polyclonal antibody to capture the antigen and MAb ¹²⁵I-6B1 for detection. 6B1 has a single epitope in CR1 (LHR-D), unlike most other anti-CR1 MAbs.

Biospecific Interaction Analysis

Binding of native C1q to immobilized sCR1 was analyzed using a BIAcore instrument (Pharmacia). sCR1 (800 μg/ml in 10 mM citrate

[pH 4.8] buffer × 50 μl at a flow of 5 μl/min) was coupled to a CM5 sensor chip (Pharmacia) using EDC and NHS according to the manufacturer's instructions (Johnsson et al., 1991). These conditions resulted in 8852-9150 RU stably coupled in five separate immobilizations. Binding studies were performed in PBS at 25°C using a flow rate of 5 μl/min. For analysis of (C3b)₂ binding, the immobilized rsCR1 was regenerated for subsequent analysis by washing with 0.1 M sodium citrate (pH 5.0). In C1q binding studies, the CR1 was regenerated by washing with 0.5 M NaCl. Data was analyzed using BIAcore Incorporated software (BIA Evaluation and BIA Simulation, Pharmacia).

Acknowledgments

Address correspondence to A. N. W. We thank the following people: Dr. Andrea Tenner of the University of California at Irvine for sharing protocols and providing an initial lot of C1q collagen tails, which were used to compete with C1q binding to immobilized rsCR1; Drs. Una Ryan and Henry Marsh of T-Cell Sciences for providing rsCR1 and monoclonal antibodies against CR1; members of the Molecular Biology Core Facility of the Dana-Farber Cancer Institute for use of the BIAcore instrument; and Katharine Stapleton and Sally Kolodkin for excellent help with the manuscript. This work was supported by a grant (DK34028) from the National Institutes of Health and a grant to L. B. K. from the Arthritis Foundation.

Received March 11, 1997; revised July 21, 1997.

References

- Arnaut, M.A., Melamed, J., Tack, B.F., and Colten, H.R. (1981). Characterization of the human complement (C3b) receptor with a fluid phase C3b dimer. *J. Immunol.* 127, 1348-1354.
- Atkinson, J.P. (1986). Complement activation and complement receptors in systemic lupus erythematosus. *Springer Semin. Immunopathol.* 9, 179-194.
- Baker, C.J., Edwards, M.S., Webb, B.J., and Kasper, D.L. (1982). Antibody-independent classical pathway-mediated opsonophagocytosis of Type Ia, group B Streptococcus. *J. Clin. Invest.* 69, 394-404.
- Bleackley, R.C., Atkinson, E.A., Burns, K., and Michalak, M. (1995). Calreticulin: a granule-protein by default or design. *Curr. Top. Microbiol. Immunol.* 198, 145-159.
- Bowness, P., Davies, K.A., Norsworthy, P.J., Athanassiou, P., Taylor-Wiedeman, J., Borysiewicz, L.K., Meyer, P.A., and Walport, M.J. (1994). Hereditary C1q deficiency and systemic lupus erythematosus. *Q. J. Med.* 87, 455-464.
- Brown, E.J. (1989). The interaction of small oligomers of complement 3b (C3b) with phagocytes. High affinity binding and phorbol ester-induced internalization by polymorphonuclear leukocytes. *J. Biol. Chem.* 264, 6196-6201.
- Changelian, P.S., Jack, R.M., Collins, L.A., and Fearon, D.T. (1985). PMA induces the ligand-independent internalization of CR1 on human neutrophils. *J. Immunol.* 134, 1851-1858.
- Chen, A., Gaddipati, S., Hong, Y., Volkman, D.J., Peerschke, E.I.B., and Ghebrehwet, B. (1994). Human T cells express specific binding sites for C1q. Role in T cell activation and proliferation. *J. Immunol.* 153, 1430-1440.
- Chevalier, J., and Kazatchkine, M.D. (1989). Distribution in clusters of complement receptor type one (CR1) on human erythrocytes. *J. Immunol.* 142, 2031-2036.
- Colten, H.R., and Rosen, F.S. (1992). Complement deficiencies. *Annu. Rev. Immunol.* 10, 809-834.
- Cullen, D.C., Brown, R.G., and Lowe, C.R. (1987). Detection of immuno-complex formation via surface plasmon resonance on gold-coated diffraction gratings. *Biosensors* 3, 211-225.
- Dickler, H.B., and Kunkel, H.G. (1972). Interaction of aggregated gamma globulin with B lymphocytes. *J. Exp. Med.* 136, 191-196.
- Edberg, J.C., Wright, E., and Taylor, R.P. (1987). Quantitative analyses of the binding of soluble complement-fixing antibody/dsDNA

- immune complexes to CR1 on human red blood cells. *J. Immunol.* 139, 3739.
- Eggleton, P., Lieu, T.S., Zappi, E.G., Sastry, K., Coburn, J., Zaner, K.S., Sontheimer, R.D., Capra, J.D., Ghebrehiwet, B., and Tauber, A.I. (1994). Calreticulin is released from activated neutrophils and binds to C1q and mannan-binding protein. *Clin. Immunol. Immunopath.* 72, 405-409.
- Fearon, D.T. (1980). Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. *J. Exp. Med.* 152, 20-30.
- Fearon, D.T., and Wong, W.W. (1983). Complement ligand-receptor interactions that mediate biological responses. *Annu. Rev. Immunol.* 1, 243-250.
- Gertler, A., Grosclaude, J., Strasburger, C.J., Nir, S., and Djane, J. (1996). Real-time kinetic measurements of the interactions between lactogenic hormones and prolactin-receptor extracellular domains from several species support the model of hormone-induced transient receptor dimerization. *J. Biol. Chem.* 271, 24482-24491.
- Ghebrehiwet, B. (1988). Reversible biotinylation of C1q with a cleavable biotinyl derivative. *J. Immunol. Meth.* 110, 251-260.
- Ghebrehiwet, B., and Hamburger, M. (1982). Purification and partial characterization of a C1q inhibitor from the membranes of human peripheral blood lymphocytes. *J. Immunol.* 129, 157-162.
- Ghebrehiwet, B., and Peerschke, E.I. (1993). The C1q-R participates in immunoregulation and signal transduction. *Behring Inst. Mitt.* 93, 236-240.
- Ghebrehiwet, B., Peerschke, E.I.B., Hong, Y., Munoz, P., and Gorevic, P.D. (1992). Short amino acid sequences derived from C1q receptor (C1q-R) show homology with the alpha chains of fibronectin and vitronectin receptors and collagen type IV. *J. Leuk. Biol.* 51, 546-556.
- Ghebrehiwet, B., Silvestri, L., and McDevitt, C. (1984). Identification of the Raji cell membrane-derived C1q inhibitor as a receptor for human C1q. Purification and immunochemical characterization. *J. Exp. Med.* 160, 1375-1389.
- Groger, R.K., Morrow, D.M., and Tykocinski, M.L. (1989). Directional antisense and sense cDNA cloning using Epstein-Barr virus episomal expression vectors. *Gene* 87, 285-294.
- Guan, E., Burgess, W.H., Robinson, S.L., Goodman, E.B., McTigue, K.J., and Tenner, A.J. (1991). Phagocytic cell molecules that bind the collagen-like region of C1q. *J. Cell. Biol.* 266, 20345-20355.
- Hammer, C.H., Wirtz, G.H., Renfer, L., Gresham, H.D., and Tack, B.F. (1981). Large scale isolation of functionally active components of the human complement system. *J. Biol. Chem.* 256, 3995-4006.
- Hebert, L.A. (1991). The clearance of immune complexes from the circulation of man and other primates. *Am. J. Kidney Dis.* 17, 352-361.
- Hourcade, D., Holers, V.M., and Atkinson, J.P. (1989). The regulators of complement activation (RCA) gene cluster. *Adv. Immunol.* 45, 381-418.
- Jack, R.M., Ezzell, R.M., Hartwig, J., and Fearon, D.T. (1986). Differential interaction of the C3b/C4b receptor and MHC class I with the cytoskeleton of human neutrophils. *J. Immunol.* 137, 3996-4003.
- Jack, R.M., Lowenstein, B.A., and Nicholson-Weller, A. (1994). Regulation of C1q receptor expression on human polymorphonuclear leukocytes. *J. Immunol.* 153, 262-269.
- Jiang, H., Siegel, J.N., and Gewurz, H. (1991). Binding and complement activation by C-reactive protein via the collagen-like region of C1q and inhibition of these reactions by monoclonal antibodies to C-reactive protein and C1q. *J. Immunol.* 146, 2314-2330.
- Johnsson, B., Lofas, S., and Lindquist, G. (1991). Immobilization of proteins to a carboxymethyl-dextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* 198, 268-277.
- Jourmet, A., and Tosi, M. (1986). Cloning and sequencing of full-length cDNA encoding the precursor of human complement component C1r. *Biochem. J.* 240, 783-787.
- Kishore, U., Sontheimer, R.D., Sastry, K.N., Zaner, K.S., Zappi, E.G., Hughes, G.R., Khamashta, M.A., Strong, P., Reid, K.B.M., and Eggleton, P. (1997). Release of calreticulin from neutrophils may alter C1q-mediated immune function. *Biochem. J.* 322, 543-550.
- Klickstein, L.B., Bartow, T.J., Miletic, B., Rabson, L.D., Smith, J.A., and Fearon, D.T. (1988). Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1, CD35) by deletion mutagenesis. *J. Exp. Med.* 168, 1699-1717.
- Klickstein, L.B., Wong, W.W., Smith, J.A., Weis, J.H., Wilson, J.G., and Fearon, D.T. (1987). Human C3b/C4b receptor (CR1). Demonstration of long homologous repeating domains that are composed of the short consensus repeats characteristic of C3/C4 binding proteins. *J. Exp. Med.* 165, 1095-1112.
- Korb, L.C., and Ahearn, J.M. (1997). C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes. *J. Immunol.* 158, 4525-4528.
- Krych, M., Hourcade, D., and Atkinson, J.P. (1991). Sites within the complement C3b/C4b receptor important for the specificity of ligand binding. *Proc. Natl. Acad. Sci. USA* 88, 4353-4357.
- Lachmann, P.J. (1984). Inherited complement deficiencies. *Philos. Trans. R. Soc. Lond. (Biol.)* 306, 419-430.
- Lepow, I.H., Naff, G.B., Todd, E.W., Pensky, J., and Hinz, C.F.J. (1963). Chromatographic resolution of the first component of human complement into three activities. *J. Exp. Med.* 117, 983-1008.
- Leytus, S.P., Kurachi, K., Sakariassen, K.S., and Davie, E.W. (1986). Nucleotide sequence of the cDNA coding for human complement C1r. *Biochemistry* 25, 4855-4863.
- Liberti, P.A., Bausch, D.M., and Baillie, R.D. (1981). The incorporation of high pH euglobulin precipitation in the isolation of C1q. *J. Immunol. Meth.* 40, 243-245.
- Lozada, C., Levin, R.I., Hule, M., Hirschhorn, R., Naime, D., Whitlow, M., Recht, P.A., Golden, B., and Cronstein, B.N. (1995). Identification of C1q as the heat-labile serum cofactor required for immune complexes to stimulate endothelial expression of the adhesion molecules E-selectin and intercellular and vascular cell adhesion molecules 1. *Proc. Natl. Acad. Sci. USA* 92, 8378-8382.
- Malhotra, R., Reid, K.M.B., and Sim, R. (1988). Studies on the isolation of human C1q-receptor. *Biochem. Soc. Trans.* 16, 735-736.
- Malhotra, R., and Sim, R.B. (1989). Chemical and hydrodynamic characterization of the human leucocyte receptor for complement subcomponent C1q. *Biochem. J.* 262, 625-631.
- Malhotra, R., Willis, A.C., Jensenius, J.-C., Jackson, J., and Sim, R.B. (1992). Structure and homology of human C1q receptor (collectin receptor). *Immunol.* 78, 341-348.
- Matsumoto, A.K., Martin, D.R., Carter, R.H., Klickstein, L.B., Ahearn, J.M., and Fearon, D.T. (1993). Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocytes. *J. Exp. Med.* 178, 1407-1417.
- Nepomuceno, R.R., Henschen-Edman, A.H., Burgess, W.H., and Tenner, A.J. (1997). cDNA cloning and primary structure analysis of C1qR, the human C1q/MBL/SPA receptor that mediates enhanced phagocytosis in vitro. *Immunity* 6, 119-129.
- Paccard, J.-P., Carpentier, J.-L., and Schifferli, J.A. (1990). Exudation induces clustering of CR1 receptors at the surface of human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* 172, 1203-1208.
- Paccard, J.P., Carpentier, J.L., and Schifferli, J.A. (1988). Direct evidence of the clustered nature of complement receptors type 1 on the erythrocyte membrane. *J. Immunol.* 141, 3889-3894.
- Peerschke, E.I.B., Malhotra, R., Ghebrehiwet, B., Reid, K.B.M., Willis, A.C., and Sim, R.B. (1993). Isolation of a human endothelial cell C1q receptor. *J. Leuk. Biol.* 53, 179-184.
- Petry, F., Berkel, A.I., and Loos, M. (1997). Multiple identification of a particular type of hereditary C1q deficiency in the Turkish population: review of the cases and additional genetic and functional analysis. *Hum. Genet.* 100, 51-56.
- Petty, H.R., Smith, L.M., Fearon, D.T., and McConnell, H.M. (1980). Lateral distribution and diffusion of the C3b receptor of complement, HLA antigens, and lipid probes in peripheral blood leukocytes. *Proc. Natl. Acad. Sci. USA* 77, 6587-6591.
- Reid, K.B.M. (1976). Isolation, by partial pepsin digestion, of the three collagen-like regions present in subcomponent C1q of the first component of human complement. *Biochem. J.* 155, 5-17.
- Reilly, B.D., Makrides, S.C., Ford, P.J., Marsh, H.C.J., and Mold, C.

(1994). Quantitative analysis of C4b dimer binding to distinct sites on the C3b/C4b receptor (CR1). *J. Biol. Chem.* 269, 7696-7701.

Shaw, S., Luce, G.G., Gilks, W.R., Anderson, K., Ault, K., Bochner, B.S., Boumsell, L., Denning, S.M., Engleman, E.G., Fleisher, T., et al. (1995). BP1.3 leucocyte differentiation antigen database. In *Leucocyte Typing V*, Schlossman, S.F., Boumsell, L., Gilks, W., Hartan, J.M., Kishimoto, T., Morimoto, C., Ritz, J., Shaw, S., Silverstein, R., Springer, T., et al., eds. (Oxford: Oxford University Press), p. 87.

Siegel, R.C., and Schumaker, V.N. (1983). Measurement of the association constants of the complexes formed between intact C1q or pepsin-treated C1q stalks and the unactivated or activated C1rC1s tetramers. *Mol. Immunol.* 20, 53-66.

Storm, D., Loos, M., and Kaul, M. (1996). Biotinylation of proteins via amino groups can induce binding to U937 cells, HL-60 cells, monocytes and granulocytes. *J. Immunol. Meth.* 199, 87-99.

Stuart, G.R., Lynch, N.J., Lu, J., Gelck, A., Moffatt, B.E., Sim, R.B., and Schwaebler, W.J. (1996). Localisation of the C1q binding site within C1q receptor/calreticulin. *FEBS Lett.* 397, 245-249.

Tenner, A.J. (1993). Functional aspects of the C1q receptors. *Behring Inst. Mitt.* 93, 241-253.

Tenner, A.J., and Cooper, N.R. (1980). Analysis of receptor-mediated C1q binding to human peripheral blood mononuclear cells. *J. Immunol.* 125, 1658-1664.

Tenner, A.J., and Cooper, N.R. (1981a). Identification of types of cells in human peripheral blood that bind C1q. *J. Immunol.* 126, 1174-1179.

Tenner, A.J., Lesavre, P.H., and Cooper, N.R. (1981b). Purification and radiolabeling of human C1q. *J. Immunol.* 127, 648-653.

Tenner, A.J., Ziccardi, R.J., and Cooper, N.R. (1984). Antibody-independent C1 activation by *E. coli*. *J. Immunol.* 133, 886-891.

Terkeltaub, R., Tenner, A.J., Kozin, F., and Ginsberg, M.H. (1983). Plasma protein binding by monosodium urate crystals. *Arthritis Rheum.* 26, 775-783.

Tosi, M., Duponchel, C., Meo, T., and Julier, C. (1987). Complete cDNA sequence of human complement C1s and close physical linkage of the homologous genes C1s and C1r. *Biochem.* 26, 8516-8524.

Tseng, Y., Zavodszky, P., and Schumaker, V.N. (1997). The human complement C1 complex has a picomolar dissociation constant at room temperature. *J. Immunol.* 158, 937-944.

Tuveson, D.A., Ahearn, J.M., Matsumoto, A.K., and Fearon, D.T. (1991). Molecular interactions of complement receptors on B lymphocytes: a CR1/CR2 complex distinct from CR2/CD19 complex. *J. Exp. Med.* 173, 1083-1089.

Uwatoko, S., and Mannick, M. (1990). The location of binding sites on C1q for DNA. *J. Immunol.* 144, 3484-3488.

Vanguri, P., Koski, C.L., Silverman, B., and Shin, M.L. (1982). Complement activation by isolated myelin: activation of the classical pathway in the absence of myelin-specific antibodies. *Proc. Natl. Acad. Sci. USA* 79, 3290-3294.

Wysocki, L.J., and Sato, V.L. (1978). "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA* 75, 2844-2848.

Ying, S.C., Gewurz, A.T., Jiang, H., and Gewurz, H. (1993). Human serum amyloid P component oligomers bind and activate the classical complement pathway via residues 14-26 and 76-92 of the A chain collagen-like region of C1q. *J. Immunol.* 150, 169-176.

Yoon, S., and Fearon, D.T. (1985). Characterization of a soluble form of the C3b/C4b receptor (CR1) in human plasma. *J. Immunol.* 134, 3332-3338.

Zhang, S.C., Schultz, D.R., and Ryan, U.S. (1986). Receptor-mediated binding of C1q on pulmonary endothelial cells. *Tissue Cell* 18, 13-18.

STIC-ILL

mic only

From: Romeo, David
Sent: Monday, June 04, 2001 1:46 PM
To: STIC-ILL
Subject: 09503421 Abrass C K; Border W A; Glassock R J

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Abrass C K; Border W A; Glassock R J

~~TI Circulating immune complexes in rats with autologous immune~~
complex nephritis.

SO LABORATORY INVESTIGATION, (1980 Jul) 43 (1) 18-27.

Journal code: KZ4; 0376617. ISSN: 0023-6837.

AN 80230958

Circulating Immune Complexes in Rats with Autologous Immune Complex Nephritis

CHRISTINE K. ABRASS, M.D., WAYNE A. BORDER, M.D., AND RICHARD J. GLASSOCK, M.D.

The Division of Nephrology and Hypertension, Department of Medicine, Harbor-UCLA Medical Center, Torrance, California 90509

Autologous immune complex nephritis (Heymann nephritis) was actively induced in rats by immunization with high (10 mg.) and low (1 mg.) doses of renal tubular epithelial antigen in complete Freund's adjuvant. The development of proteinuria and granular capillary wall deposition of IgG confirmed the previously well described membranous nephropathy which characterizes this experimental disease. Circulating immune complexes were demonstrated by both the fluid phase and solid phase C1q binding assays in both high and low dose experimental groups. The prevalence of such immune complexes was significantly greater in the experimental than in the control groups immunized with adjuvant alone or liver homogenate in adjuvant. The circulating immune complexes bound to C1q were 16 to 23 S in size and were proven to contain a renal tubular antigen. These data, in combination with the previous demonstration of renal tubular antigen and its antibody in kidneys from rats with autologous immune complex nephritis, are consistent with a circulating immune complex pathogenesis of this model of the actively induced autologous immune complex nephritis in rats.

Additional key words: Heymann nephritis, Renal tubular epithelial antigen, Membranous nephropathy.

In 1959, Heymann and co-workers (12) first described an experimental model of membranous nephropathy which was induced by immunization of rats with homologous kidney in adjuvant. The pathogenic mechanism was subsequently evaluated by Edgington, Glasscock, and Dixon (6,8,10). They isolated an antigen, designated RTE- α_5 , from the proximal renal tubule brush border which when given to rats in adjuvant produced the disease. Antibody reactive with the RTE antigen was demonstrated in the circulation (10) and immunoglobulin, complement, and RTE were demonstrated in subepithelial deposits in the glomerulus (6). Antibody eluted from the kidney was specific for the renal tubular antigen. Utilizing human RTE- α_5 to induce the disease in rats and species-specific antibody to demonstrate its presence in tissue deposits, Edgington, Glasscock and Dixon (7) demonstrated both immunizing antigen and autologous RTE antigen in the kidney. Based on these observations and the pattern of immunoglobulin deposits, an immune complex pathogenesis was proposed. It was suggested that the immunizing antigen initiated autoantibody production through termination of natural tolerance. Antibody binding to endogenously released antigen resulted in circulating immune complexes which deposited in the glomeruli and produced the glomerular lesion. The term autologous immune complex nephritis (AICN) was coined to describe these pathogenic events (6,8,10).

The circulating immune complex pathogenesis of AICN in rats was widely accepted until recently, when

additional studies involving passive transfer of antibodies to RTE initiated controversy. Sugisaki and co-workers (23) demonstrated passive transfer of disease with homologous IgG from animals with actively induced disease. First Barabas, Nagi, and Lannigan (1) and later Feenstra *et al.* (9) were able to induce a lesion similar to or identical with that produced by active immunization by injecting heterologous antiserum produced to Fx1A, a crude preparation of the renal tubular epithelial antigen. Van Damme *et al.* (24) showed early fixation of heterologous antibody to presumed subepithelial glomerular antigens within 1 to 3 hours postinfusion. Couser *et al.* (5) showed early fixation of heterologous anti-Fx1A antibody in the subepithelial space in the isolated perfused rat kidney, thus eliminating the possibility of formation of circulating immune complexes. Based on these findings, Van Damme and colleagues (24) and Couser and co-workers (5) have proposed that the immunoglobulin deposits result from antibody binding to fixed glomerular antigens. Demonstration of such specificity of the heterologous antibody produced to Fx1A suggested the possibility of a similar mechanism in the actively induced model. The passively induced models are known as heterologous immune complex nephritis or passive Heymann nephritis.

There were two reasons for embarking on the following investigation. An animal model of membranous nephropathy provided the opportunity to investigate the prevalence and immunochemical characteristics of presumably

pathogenic immune complexes. Demonstration and characterization of immune complexes in AICN would help to clarify the controversy regarding the pathogenesis of this animal model. The present study evaluates the incidence of circulating immune complexes (CIC) in rats with actively induced autologous immune complex or Heymann nephritis. Immune complexes are measured by both the fluid and solid phase C1q binding radioimmune assays. In addition, size characteristics and antigen composition of the detected immune complex are presented.

MATERIALS AND METHODS

ANTIGENS

Kidneys and livers were removed from 20 normal Sprague-Dawley rats (Microbiological Associates, Bethesda, Maryland) and washed free of blood with cold isotonic saline. Kidney cortex was minced and renal tubular epithelial antigen was prepared as fraction 1A (FxIA) by the method of Edgington, Glasscock and Dixon (7). The FxIA was lyophilized and stored at -70°C . Liver was minced, suspended in two volumes of cold isotonic saline, and homogenized for 2 minutes at full speed in a Sorvall Omnimixer. The homogenate was sedimented and washed three times with distilled water by centrifugation each time ($27,000 \times g$ at 4°C for 30 minutes). The liver homogenate was lyophilized and stored at -70°C . Bovine serum albumin (BSA) was purchased from Miles Laboratories Inc. (Elkhart, Indiana).

EXPERIMENTAL GROUPS

Immunizations. Male Lewis rats weighing 200 to 250 gm. were used exclusively (Microbiological Associates, Bethesda, Maryland). Antigens were suspended in complete Freund's adjuvant (CFA) containing 4 mg. of mycobacterium tuberculosis (Difco Laboratories, Detroit, Michigan, H37 RA) per milliliter of adjuvant. Each animal was immunized once with 0.25 ml. of material divided equally between the hind footpads. Animals were divided into six groups of 10 animals each. Each animal in group 1 received 10 mg. of FxIA, group 2 received 1 mg. of FxIA, groups 3 and 4 received adjuvant only, group 5 received 10 mg. of liver homogenate, and group 6 received 10 mg. of BSA.

EXPERIMENTAL PROCEDURES

The following determinations were made in all animals: Prior to immunization and at weekly intervals thereafter until sacrifice at 14 weeks, animals were housed for 24 hours in metabolic cages without food, but water was provided *ad libitum*. Twenty-four hour-urine collections were analyzed for protein by the method of Kingsbury and Clark (13).

Approximately 1.5 ml. of blood was collected weekly from the tail vein of each rat. Serum was isolated by centrifugation at room temperature (1200 r.p.m., 10 minutes) and stored at -70°C . Antibody determinations were performed on all sera. Circulating antibody to renal tubular epithelial antigen (RTE) was determined by indirect immunofluorescence using cryostat sections from normal Lewis kidney and fluorescein-conjugated rabbit anti-

rat IgG (Cappel Laboratories, Inc., Cochranville, Pennsylvania). Antibody was determined by serial 2-fold dilutions of serum, and the titer was defined as the highest dilution which exhibited positive fluorescent staining of the proximal tubular brush border. Circulating antibody to liver antigen was searched for by indirect immunofluorescence using cryostat sections of normal Lewis liver. Antibody to BSA was detected by double-diffusion in agarose gel and semiquantitated from 0 to 4 plus.

C1q BINDING ASSAYS

Samples selected for determination on any given assay day were chosen at random from the stored samples. Twenty per cent of all samples were randomly selected for assay on more than one assay day. With rare exceptions due to insufficient quantity, all samples were analyzed in duplicate in both assays.

REAGENTS

C1q was isolated from normal fresh human serum by the method of Yonemasu and Stroud (30). Protein concentration of C1q was determined by the method of Lowry *et al.* (15) using a human protein standard (Dade, Miami, Florida). C1q was stored at -70°C prior to use. Aggregated rat γ -globulin (ARG) was prepared by heating 5 ml. of a 2 per cent solution of rat IgG (Miles Laboratories, Inc., Elkhart, Indiana) in phosphate-buffered saline (PBS) at 63°C for 30 minutes. The preparation was cooled to room temperature and insoluble aggregates were removed by centrifugation at $5000 \times g$, at 4°C for 30 minutes. Monomeric IgG was not removed from the solution; thus, the total protein concentration of the preparation represents both aggregated and monomeric IgG. The ARG preparation was aliquoted and stored at -70°C . The same preparation of ARG was used throughout the study. Protein concentration was determined by the biuret method (26), using a human protein standard. Rabbit anti-rat IgG was produced by hyperimmunization of New Zealand White rabbits with rat IgG (Miles Laboratories, Inc., Elkhart, Indiana) in CFA. Antiserum was tested for specificity by immunoelectrophoresis. Rabbit γ -globulin was precipitated with 50 per cent saturated ammonium sulfate, resuspended in PBS, and stored at -70°C prior to use. IgG concentration was estimated from optical density measurements at 280 nm. $E_{280}^{1\%} = 14.6$.

Proteins (C1q and rabbit anti-rat IgG) were radioiodinated with I^{125} by the chloramine-T method of McConeahey and Dixon (17) and stored at -70°C in 1 per cent BSA-PBS. Trichloroacetic acid-precipitable accounts were >95 per cent of the total. Gelatin (Difco Laboratories, Detroit, Michigan) at a concentration of 0.1 per cent was substituted for BSA in all solutions used for CIC determinations on the sera of rats from group 6 which had been immunized with BSA.

FLUID PHASE C1q BINDING ASSAY (FC1q)

This assay was modified from the method of Nydegger *et al.* (19). Test serum (200 μl .) was added to 100 μl . of Veronal-buffered saline (VBS) and heated at 56°C for 30 minutes and then cooled to room temperature. ^{125}I -C1q (0.5 μg .) in 100 μl . of 1 per cent BSA-VBS (0.1 per

cent gelatin-VBS for group 6 samples) which had been centrifuged at $7800 \times g$ at 4°C . for 30 minutes just prior to use was added to the reaction mixture and incubated at 37°C . for 30 minutes. The immune complex material was twice precipitated with 2.5 per cent polyethylene glycol (PEG) (molecular weight 6000, JT Baker Chemical, Phillipsburg, New Jersey) at 4°C . The precipitate was isolated by centrifugation ($1500 \times g$, 4°C ., 30 minutes), and the radioactivity counted in an automatic Beckman 7000 gamma counter. Serial dilutions of ARG from 10 to 5000 μg . per ml. in VBS added to an aliquot of a normal Lewis-Sprague-Dawley rat serum pool were assayed as above and used to generate a standard curve. The means of duplicate determinations of test sera were calculated as a per cent of maximal ARG binding and expressed as micrograms per milliliter of ARG equivalents. The FC1q assay detected as little as 30 to 50 μg . per ml. of ARG in normal rat serum. The intraassay and interassay coefficients of variation were 3.56 per cent and 8.08 per cent, respectively.

SOLID PHASE C1q BINDING ASSAY (SC1q)

This assay was modified from the method of Hay, Nineham, and Roitt (11). Each 12-by 75-mm. polystyrene tube (Falcon, Oxnard, California) was incubated at 4°C . for 20 hours with 1 ml. of a 5- μg . per ml. PBS solution of freshly isolated human C1q. The tubes were washed with PBS, incubated for 2 hours at room temperature with 1.5 ml. of 1 per cent BSA-PBS (or gelatin-PBS for group 6 samples), washed again with PBS, and stored at -70°C . Fifty microliters of test serum were added to 100 μl . of 0.2 M ethylenediaminetetraacetic acid (EDTA), pH 7.4, and incubated at 37°C . for 30 minutes. Sixty microliters of this solution were added to 940 μl . of PBS into a C1q-coated tube and incubated at 37°C . for 1 hour and at 4°C . for 20 hours, and then washed three times with PBS. ^{125}I rabbit-antirat IgG (3.5 μg .) in 1 ml. of 1 per cent BSA-PBS (or 0.1 per cent gelatin-PBS for group 6 samples) was added into the tube and incubated at 37°C . for 1 hour and at 4°C . for 30 minutes and washed three times with PBS and counted in an automatic gamma counter. ARG in serial dilutions from 1 to 750 μg . per ml. suspended in an aliquot of a normal rat serum pool was assayed as above to produce a standard curve. The means of duplicates of test sera were calculated as a per cent of maximal ARG binding and expressed as μg . per ml. of ARG equivalents. The SC1q assay detected as little as 1 to 5 μg . per ml. of ARG in normal rat serum. The intraassay and interassay coefficients of variation were 2.28 per cent and 5.16 per cent, respectively.

HISTOLOGIC EVALUATION

Renal cortex obtained at sacrifice was fixed in alcoholic Bouin's solution for light microscopy. Paraffin sections (2 μm . thick) were stained with hematoxylin and eosin, Masson's trichrome, and Jones silver-methenamine stains. Renal tissue in isopentane was snap-frozen with liquid nitrogen for immunofluorescence. Cryostat sections (2 μm .) were incubated with fluorescein-conjugated rabbit-antirat IgG (Cappel Laboratories, Cochranville, Pennsylvania), washed, and examined with a Leitz transmissi n immunofluorescence microscope.

EVALUATION OF POLYETHYLENE GLYCOL (PEG) PRECIPITATES

Gradient Ultracentrifugation. The size of C1q-reactive material was analyzed by ultracentrifugation on a linear 10 to 40 per cent sucrose gradient. Two-hundred microliters of test serum in 100 μl . of VBS were heated at 56°C . for 30 minutes. One-hundred nanograms of ^{125}I -C1q in 100 μl . of 1 per cent BSA-VBS (or gelatin-VBS) prepared as for the FC1q assay, were added to the reaction mixture and incubated at 37°C . for 30 minutes. Three-hundred-fifty microliters were layered on the sucrose gradient in 13-ml. capacity tubes and centrifuged in a SW-40TI rotor at 38,000 r.p.m. ($180,000 \times g$) for 20 hours. Three drop fractions were collected and counted for radioactivity in an automatic gamma counter. Sedimentation markers included I^{131} -labeled BSA and human thyroglobulin. Each fraction was then studied by double immunodiffusion utilizing rabbit antirat IgG to determine those fractions which contained rat IgG. Twelve experimental samples, two samples of normal rat serum, and two samples of normal rat serum containing ARG were analyzed as above. The experimental samples including six samples from group 1 and three samples from group 3 that were positive in one of the immune complex assays and two samples from group 1 and one sample from group 3 that had no detectable immune complexes.

Evaluation of Anti-RTE Antibody. PEG precipitates were prepared as above for the FC1q assay, redissolved in 100 μl . of PBS, and analyzed for binding to proximal tubular brush border by indirect immunofluorescence. Eight samples from groups 1 and 2 and four samples from group 3 and 4 were tested. Antibody titer relative to IgG concentration was not determined.

Evaluation of RTE Antigen. The polyethylene glycol (PEG) precipitate of a FC1q positive, day 70 serum from an experimental animal (immunized with Fx1A), and one from a control animal (immunized with CFA only) were redissolved in 100 μl . of PBS and suspended in CFA. Two New Zealand White rabbits were given a primary immunization with the PEG precipitate preparations followed by a booster injection 14 days later. Antisera were collected at 10 days after the primary injection and at 7 days after the booster injection. Rabbit antibody binding to renal tubular epithelium was evaluated by indirect immunofluorescence with *in vitro* complement fixation (21) utilizing fresh normal human serum as a complement source. The sections were stained with fluorescein-conjugated goat-antihuman C3 (Meloy Laboratories, Springfield, Virginia), and the pattern of fluorescence was observed. Controls included direct fluorescence with the conjugated antihuman-C3; and indirect immunofluorescence with normal human serum, normal rabbit serum, normal rat serum, and rabbit antisera which had been absorbed with normal rat serum, rat liver homogenate, rat Fx1A, and rat glomerular basement membrane (prepared by the method of Schoenfeld and Glassock (21)). A normal human serum sample that failed to show any binding to rat proximal tubule was selected, and the same human serum sample was used throughout the above described procedure.

In addition to the above, PEG precipitates from 200 μl . of FC1q-positive samples from three group 1, two

group 2, three group 3, and two group 4 samples were prepared as described above. Each PEG precipitate was redissolved in 100 μ l. of PBS, suspended in CFA to a total volume of 0.25 ml., and injected into the hind footpads of male Lewis rats. Blood was collected from the tail vein for anti-RTE antibody testing by indirect immunofluorescence at 3, 4, and 8 weeks after immunization. Unilateral nephrectomy was performed on each animal at 4 and 8 weeks after immunization. Cryostat sections were prepared for immunofluorescence observation as described above.

STATISTICAL ANALYSIS

The prevalence of serum positivity for C1q-reactive material in the experimental groups was compared to the control groups by χ^2 analysis. The frequency of positivity for C1q-reactive material per animal in the experimental group was compared to the control groups by Student's *t*-test. Each animal was assigned a frequency of positivity score based on the number of times each animal had detectable C1q-reactive material by one or both assay techniques. Correlation between assay techniques was analyzed by the χ^2 test.

RESULTS

DOCUMENTATION OF EXPERIMENTAL GLOMERULONEPHRITIS

Proteinuria. The mean value \pm 2 standard deviation (SD) for protein excretion of all animals prior to immunization was 2.97 ± 2.72 mg. per 24 hours. Thus, a 24-hour protein excretion of >5.70 mg. was considered abnormal. All animals in groups 1 and 2 had developed abnormal protein excretion by day 35. No animal in groups 3, 4, 5, or 6 developed abnormal protein excretion.

Histology. By immunofluorescence observation all animals in groups 1 and 2 had granular deposits of rat IgG along the glomerular capillary wall (Fig. 1). Those animals with more pronounced IgG deposition also showed "spike" formation of basement membrane by the Jones stain and subepithelial protein deposits by trichrome stain of sections prepared for light microscopic examination. No animal in groups 3, 4, 5, or 6 had IgG deposits by immunofluorescence or abnormalities by light microscopy.

Free Antibody Determination. Sera (130, 10 preimmunization and 120 postimmunization) were analyzed for each group of animals. All animals in groups 1 and 2 and no animals in groups 3, 4, 5, or 6 produced antibody which bound to the proximal tubular brush border as evidenced by indirect immunofluorescence. The circulating antibody produced by animals in groups 1 and 2 reacted with the proximal tubular brush border only and not glomeruli at all dilutions studied (Fig. 2). In most animals circulating antibody to RTE was first detectable at day 7, peak titers (usually 1:256) occurred at days 49 to 63 and subsequently slowly declined. Antibody reactive with renal tubular and glomerular cell nuclei was observed in zero of 130 sera from group 1, zero of 130 from group 2, seven of 130 sera from group 3, zero of 130 from group 4, 11 of 130 from group 5, and zero of 130 from group 6. None were present in a titer of greater than

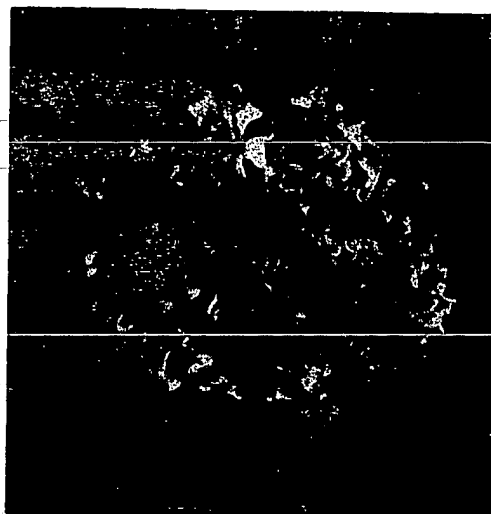


FIG. 1. Immunofluorescence photomicrograph utilizing fluorescein-conjugated rabbit anti-rat IgG on a cryostat section of a Group I kidney demonstrating granular deposition of rat IgG along the glomerular capillary wall. $\times 500$.

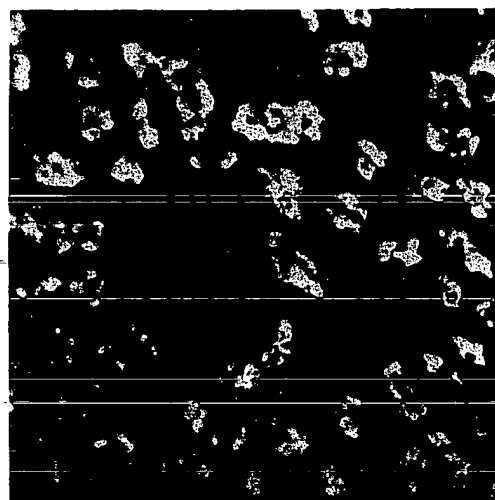


FIG. 2. Indirect immunofluorescent micrograph with serum from a Group I animal on a normal rat kidney section, stained with fluorescein-conjugated rabbit anti-rat IgG. Positive fluorescence of the brush border of the proximal renal tubule and negative glomeruli. $\times 312$.

1:4. There was no correlation between the presence of C1q-reactive material and circulating antibody reactive with nuclear material. All animals in group 6 made antibody to BSA. Low levels of free antibody were detectable 14 days postimmunization with BSA, peaked days 56 to 70, and slowly decreased thereafter. No animal in group 5 produced antibody reactive with liver antigens that were detectable by indirect immunofluorescence.

DEMONSTRATION OF C1q BINDING MATERIAL

Preimmunization Sera. Preimmunization sera from 60 male Lewis rats were analyzed by both the FC1q and SC1q assays. The upper limit of normal for each assay was arbitrarily established as 3 SD above the mean value for all preimmunization sera. The mean value $+3$ SD

(8.48 + 12.0 per cent) for the FC1q assay was 20.5 per cent of maximal ^{125}I -C1q bound. This corresponded to the activity generated by 100 μg . per ml. of ARG. The mean value ± 3 SD (32.8 + 10.7 per cent) for the SC1q assay was 53.2 per cent of maximal ^{125}I anti-IgG bound. This corresponded to the activity generated by 11 μg . per ml. of ARG. Utilizing these limits <5 per cent (zero of 60) of preimmunization sera were abnormal.

Prevalence of C1q-Reactive Material. The results of the FC1q and SC1q assays for each animal group are shown in Table 1. There was no difference between groups in the number of animals that had at least one sample positive by either assay. C1q-reactive material was more common in samples from group 1 animals than samples from group 3 animals: FC1q ($X^2 = 3.95$, $p < 0.05$), SC1q ($X^2 = 6.26$, $p < 0.025$), and either or both assays ($X^2 = 10.33$, $p < 0.005$). C1q-reactive material was also more common in samples from group 2 animals than samples from group 4 animals: FC1q ($X^2 = 12.73$, $p < 0.005$), SC1q ($X^2 = 17.42$, $p < 0.005$), and either or both assays ($X^2 = 33.86$, $p < 0.005$). C1q-reactive material was more frequent in animals from group 1 than animals from group 3: FC1q ($t = 2.54$, $p < 0.025$), either or both assays ($t = 2.40$, $p < 0.025$) and was more frequent in animals from group 2 than animals from group 4: FC1q ($t = 3.09$, $p < 0.005$), SC1q ($t = 3.58$, $p < 0.005$), either or both assays ($t = 5.05$, $p < 0.005$). There were no significant differences in C1q-reactive material between animals from groups 3, 4, and 5. One hundred of 120 samples from animals in group 6 had C1q-reactive material by one or both assays. Thus, immunization with BSA, subsequent demonstration of antibody to BSA, and positive C1q binding assays provided a positive control for the serologic tests. There was a wide range in the binding activity by both assays in all groups (Figs. 3a and b). Although all animals in groups 1 and 2 had C1q-reactive material present in at least one sample (mean frequency per animal in group 1 = 4.9; group 2 = 6.4), the level of reactivity fluctuated in time throughout the study period. There was no correlation between results in the FC1q and SC1q assays, although some samples were positive

in both assays. In animals from groups 1 and 2, free antibody was detectable 7 to 14 days postimmunization and persisted throughout the study period. CIC were detectable in some animals as early as 7 days postimmunization and were detected throughout the entire study period. Other investigators (10) have demonstrated that glomerular IgG deposition is demonstrable 3 to 4 weeks postimmunization and gradually accumulates thereafter. Thus, the time course of serologic results is consistent with the glomerular findings, but it does not favor a given pathogenic mechanism.

CHARACTERIZATION OF C1q-REACTIVE MATERIAL

Size. Sucrose gradient ultracentrifugation revealed that normal rat serum demonstrated only the 11.2 S peak of monomeric C1q. By double immunodiffusion IgG was only present in the 7 S fractions. Normal rat serum that contained ARG demonstrated C1q binding in a broad peak ranging from 14 S to 25 S. Rat IgG was also demonstrated in those fractions. The two samples from group 1 and one sample from group 3 with negative C1q binding assay results, and the three samples from group 3 with positive C1q binding results had sucrose gradient patterns indistinguishable from normal rat serum (Fig. 4A). The six samples from group 1 (four FC1q-positive and two SC1q-positive) demonstrated heavy sedimenting ^{125}I -C1q of >14 S with a 19 to 20 S peak. The heavy sedimenting peaks also contained rat IgG. Representative patterns are shown in Figure 4C and D. Serum positive in the FC1q assay (binding activity, 88.5 per cent) from a group 6 animal 21 days postimmunization had a similar pattern (Fig. 4B). The demonstrated peaks represent the sedimentation coefficients of the material that has incorporated C1q into the macromolecular complex. It does not necessarily reflect the exact molecular weight of the material *in vivo*.

DEMONSTRATION OF ANTI-RTE ANTIBODY IN C1q-REACTIVE MATERIAL

Seven of eight PEG precipitates from samples from group 1 and group 2 animals demonstrated binding to th

TABLE 1. SUMMARY OF FLUID AND SOLID PHASE C1q BINDING ASSAY RESULTS^a

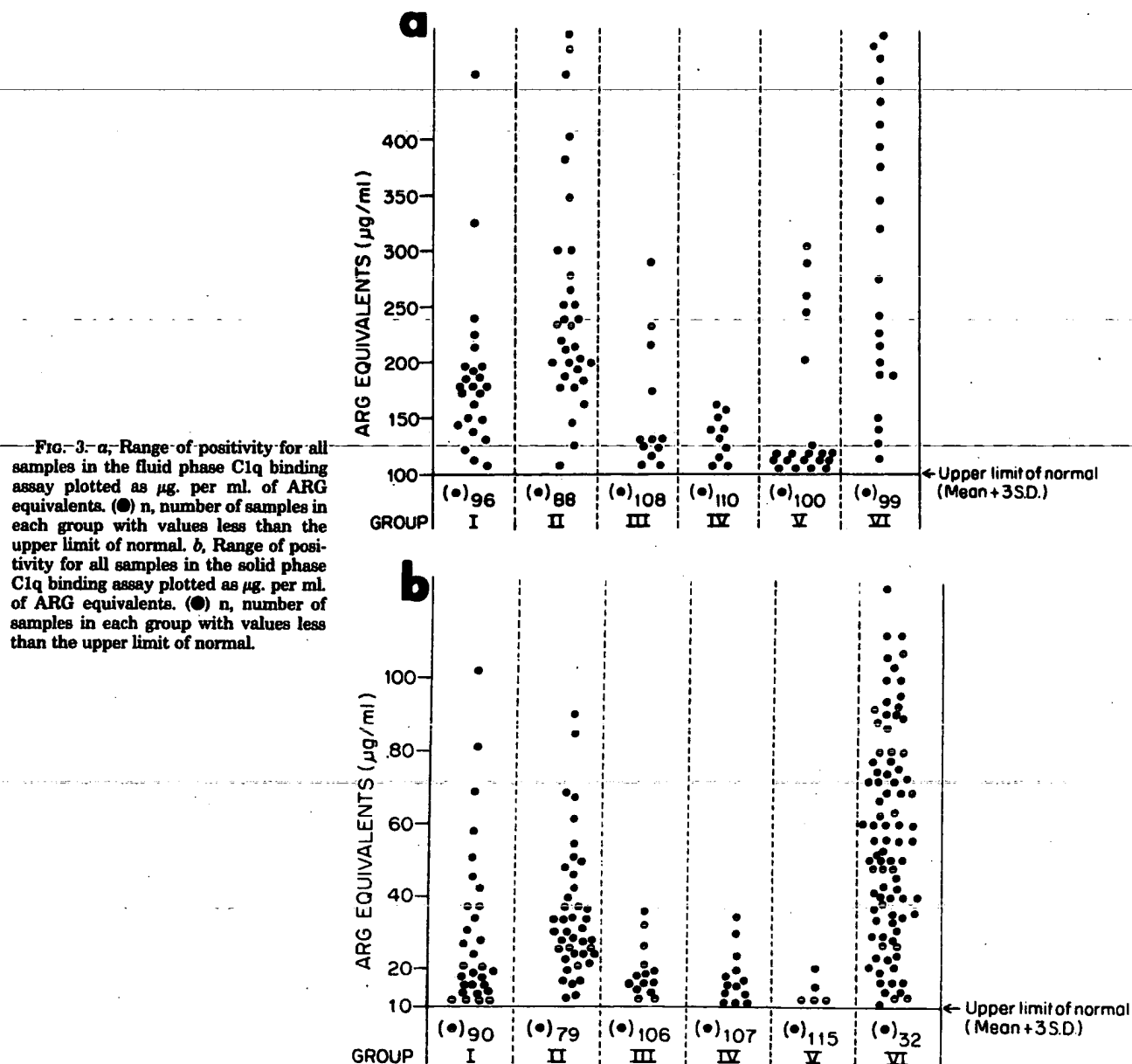
	I FxlA (10 mg.)/CFA	II FxlA (1 mg.)/CFA	III CFA alone	IV CFA alone	V Liver homogenate (10 mg.)/CFA	VI BSA (10 mg.)/CFA
Animals positive ^b (n = 10)	10	10	8	10	6	10
Samples positive ^b (n = 120)						
FC1q	24 ^c	32 ^d	12	10	20	21
SC1q	30 ^c	41 ^d	14	13	5	87
Either/both	49 ^d	64 ^d	25	20	22	100
Mean frequency of positivity score /animal						
FC1q	2.4 ^c	3.2 ^d	1.2	1.0	2.0	2.1
SC1q	3.0	4.1 ^d	1.4	1.3	0.5	8.7
Either/both	4.9 ^c	6.4 ^d	2.5	2.0	2.2	10

^a Roman numerals indicate animal group. Dose of immunogen is given in parentheses.

^b Positive at any time.

^c $p < 0.05$, different from control, group I versus III.

^d $p < 0.005$, group II versus IV.



proximal tubular brush border and no binding to the glomerulus. Four of four PEG precipitates from group 3 animals failed to show any binding to renal cortical structures when studied by indirect immunofluorescence. Five serum samples from group 1 which were negative in the FC1q assay failed to show a visible precipitate.

DEMONSTRATION OF RTE ANTIGEN IN C1q-REACTIVE MATERIAL

Rabbit antiserum produced by immunization with the PEG precipitate of a group 1 serum collected 70 days postimmunization with Fx1A bound to the brush border of the proximal renal tubule as demonstrated by amplified indirect immunofluorescence with *in vitro* complement fixation (Fig. 5a). The pattern of binding was the same as that observed by the homologous rat anti-RTE

as well as rabbit antiserum produced to rat Fx1A. No fluorescence was observed when these antisera were substituted with normal human serum, normal rabbit serum, or fluorescein conjugate alone. Absorption studies confirmed the specificity for the RTE antigen of the rabbit antiserum to the PEG precipitate of a group 1 animal (Table 2). Antiserum to the PEG precipitate from a group 3 animal failed to show specificity for any renal or liver component by indirect immunofluorescence or rat IgG as tested by double immunodiffusion.

PEG precipitates which were redissolved, suspended in CFA, and injected into male Lewis rats were all collected from experimental (groups 1 and 2) or control (groups 3 and 4) animals 49 to 84 days after their primary immunization. All had significant binding activity in the FC1q assay ranging from 35 to 70 per cent. Serum collected from rats 3, 4, and 8 weeks after immunization

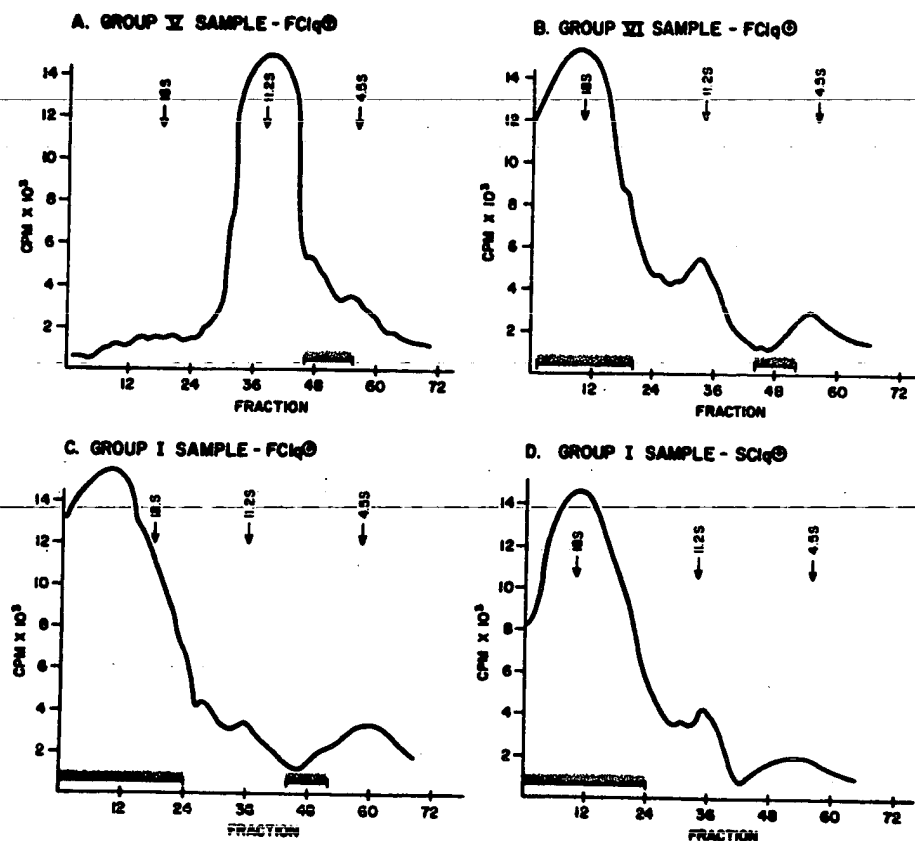


FIG. 4. Patterns of sucrose gradient ultracentrifugation analysis. Amount of ¹²⁵I C1q (expressed as c.p.m. × 10³) plotted as a function of fraction number. Dotted area, fractions which contained rat IgG.

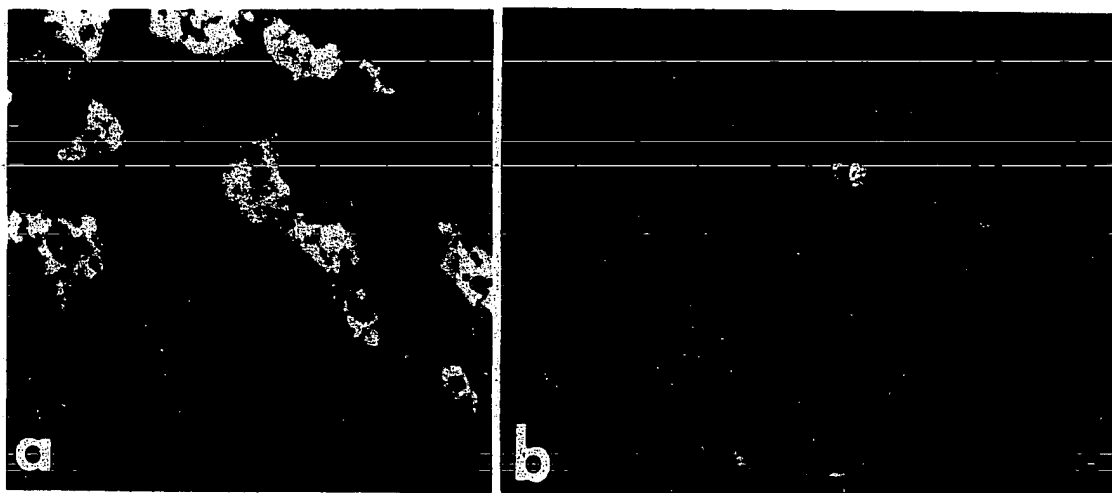


FIG. 5. *a*, Positive staining of the brush border of the rat proximal renal tubule with rabbit anti-PEG precipitate (Group I) by indirect immunofluorescence with *in vitro* complement fixation. *b*, Granular

deposition of rat IgG along the capillary wall of the glomerulus of a rat immunized with the PEG precipitate from a Group II animal. ×500.

with the PEG precipitates demonstrated anti-RTE antibody activity in four of five animals immunized with PEG precipitates from experimental animals and zero of five immunized with PEG precipitates from control animals. The pattern of binding to the proximal tubular brush border was indistinguishable from that demonstrated in Figures 2 and 5*a*. None of the sera bound to normal rat glomeruli. Tissue from unilateral nephrectomy at 4 weeks postimmunization revealed fine granular

glomerular capillary wall deposits in one animal in which anti-RTE antibody was also demonstrated. This same animal had abnormal proteinuria and died 5 weeks after immunization. At sacrifice, 8 weeks postimmunization two of three remaining animals with anti-RTE antibody in their serum had capillary wall deposits of rat IgG in their glomeruli (Fig. 5*b*). All six animals without circulating anti-RTE antibody had negative immunofluorescence of their kidneys.

TABLE 2. ANTIBODIES TO POLYETHYLENE GLYCOL (PEG) PRECIPITATES INDUCED IN RABBITS AND RATS: SPECIFICITY STUDIES^a

Sera tested	Unabsorbed	Absorbed with:			
		Rat GBM	Rat Fx1A	Rat liver homogenate	Normal rabbit/rat serum ^b
Normal rabbit serum	-	-	-	-	ND
Rabbit anti-PEG ^c	+	+	-	+	+
Normal rat serum	-	-	-	-	ND
Rat anti-PEG ^d	+	+	-	+	+
Rabbit antirat Fx1A ^e	+	+	-	+	+

^a Immunofluorescent demonstration of antibody binding to proximal tubular brush border.

^b ND, Not done.

^c Rabbit immunized with PEG precipitate from sera of rat immunized with Fx1A in CFA.

^d Rat immunized with PEG precipitate from sera of rat immunized with Fx1A in CFA.

^e Rabbit immunized with Fx1A in CFA.

DISCUSSION

Autologous immune complex nephritis was actively induced in 100 per cent of Lewis rats immunized with high and low dose Fx1A. Utilizing two assays, material having the characteristics of immune complexes was demonstrated in the experimental animals with a prevalence greater than the control animals. Animals immunized with BSA served as a positive control for the CIC assays as well as a reference group for prevalence and levels of CIC in an accepted model of immune complex disease. The BSA-immunized animals lacked IgG in their glomeruli at sacrifice on day 90. A single dose of an exogenous antigen may induce acute serum sickness; however, glomerular deposits are rapidly eliminated if exogenous antigen is not repeatedly administered (28). Presumably the antibody-excess-CIC detected in BSA-immunized rats 90 days after immunization were not as nephritogenic as those formed in antigen excess. This feature may reflect important contributions made by the character as well as amount of antigen. As suggested by Border and Cohen (3), antigen charge may be one of these factors.

Interpretation of the results is dependent upon the validity of the immune complex assays used. The FC1q and SC1q assays have been widely used to study human sera and were readily adapted to the evaluation of the animal sera. However, there are several apparent differences in assay performance between human and rat sera. We did not use the Zubler *et al.* (31) modification of the FC1q assay because calcium and magnesium were omitted from the buffer making it unnecessary to add EDTA. In addition, sucrose density analyses failed to show in normal rat sera significant amounts of the 16 S C1qrs complex. We have not observed significant aggregation of IgG by heat inactivation. The influence of heat inactivation and EDTA treatment of serum on the FC1q assay has been extensively evaluated by Soltis *et al.* (22). There are disadvantages with both methods and they fail to demonstrate that one method of decomplexation is superior. The background binding of normal rat serum had a mean of 8.48 per cent of maximal binding of ¹²⁵I-C1q. If this is recalculated as a per cent of total counts

added the mean is 3.76 per cent which is in keeping with published human data (20, 29). The upper limit of normal for the FC1q expressed as 100 µg. per ml. of ARG equivalents is artificially high as the total protein concentration of our preparation of ARG (unlike commonly prepared aggregated human IgG) contained large amounts of monomeric IgG as well as ARG. ARG equivalents represents a semiquantitative standard for assay activity but does not directly estimate the concentration of immune complex material in a test sample. Other C1q-binding materials (*e.g.*, heparin, DNA, endotoxin) theoretically may interfere in the FC1q assay but only in amounts unlikely to be present in animal or human sera (14).

There was no correlation between the FC1q and SC1q assay results in any of the animal groups studied. This lack of correlation has also been reported in studies employing human sera (2,14). Utilizing *in vitro* prepared complexes of tetanus toxoid-antitoxoid, Casali and co-workers (4) found the FC1q assay to be more sensitive in detecting CIC formed at relative antigen excess while the Raji cell and bovine conglutinin-binding assays were more sensitive in detecting CIC formed at relative antibody excess. The SC1q assay, like the Raji cell assay, detects CIC by the presence of IgG; thus, one would expect it to be more sensitive in detecting CIC formed at relative antibody excess. This differential sensitivity was further demonstrated by the observation that *in vitro* addition of excess BSA antigen to SC1q or FC1q-positive sera from animals immunized with BSA converted the assays to negative (C. K. Abrass, W. A. Border, R. J. Glasscock, unpublished observations). Such differential sensitivities of the FC1q and SC1q assays could account for their lack of correlation as well as changing reactivity of sera from rats with AICN.

In addition to having a higher prevalence and a greater frequency per animal, the C1q-reactive material that was detected in animals that were immunized with Fx1A had other properties of immune complexes. Results of sucrose density gradient analysis demonstrated that the material bound to C1q had a sedimentation coefficient of 14 to 20 S. As determined by double immunodiffusion this heavy sedimenting material contained rat IgG. Interestingly, assay-positive sera from animals immunized with adjuvant alone did not contain a similar peak. However, most of the radioactivity was recovered in the pellet suggesting the presence of material in excess of 30 S. These pellets did not contain rat IgG, and the exact nature of this macromolecular material is not known.

Some of the PEG precipitates from Fx1A-immunized animals contained an RTE antigen and anti-RTE antibody. The anti-RTE antibody in the PEG precipitates may in part represent free antibody that is nonspecifically precipitated. However, the simultaneous presence of C1q, RTE antigen, and anti-RTE antibody suggest the presence of an immune complex. Furthermore, immunization with the PEG precipitate led to the development of serologic, clinical, and glomerular abnormalities identical with the actively induced disease, confirming the presence of the putative nephritogenic RTE antigen in the immune complex material.

It is reasonable to assume that the material detected

in the sera of rats with AICN is an immune complex composed of an RTE antigen and its antibody. The RTE antigen in the CIC might be immunizing and/or endogenously released antigen. We have no data to resolve this issue. It seems likely, however, that early after immunization, administered antigen contributes to the formation of immune complexes. But after immune elimination occurs, administered antigen should no longer be present in the circulation. It also seems unlikely that administered antigen would be present 70 to 84 days after immunization with a low dose of Fx1A.

The data presented here document that immune complex material is present in sera from animals with actively induced AICN; however, they do not prove that CIC are responsible for the subepithelial deposits that are characteristic of this animal model of membranous nephropathy. Glasscock, Edgington and Dixon (10) purified human and rat RTE α_5 and confirmed that purified antigen produced disease identical with that of the crude preparation of kidney in adjuvant. Rats immunized with human RTE α_5 were studied with species-specific antisera. Human RTE was deposited early. After 6 weeks increasing amounts of rat RTE gradually accumulated. As with our homologous anti-RTE antibody (all dilutions of 240 sera) and the anti-PEG precipitate antibody, they found no binding of anti-RTE α_5 to glomeruli *in vitro*. These data suggest that an antigen normally extrinsic to the glomerulus is accumulating at a subepithelial site as the disease evolves. However, from the data available from the present experiments we cannot exclude the concomitant *in situ* formation of immune complexes as circulating antibody reacts with a fixed, intrinsic glomerular antigen.

It is interesting that most of the animals only have detectable CIC from 40 to 60 per cent of the time, while sequential morphologic studies demonstrate a progressive accumulation of IgG in the glomerulus. Several explanations are possible: (1) CIC are present but at levels below the sensitivity of the assay; (2) CIC detected in the circulation (14 to 20 S in size) represent those "left behind" and other CIC have deposited in the glomerulus; (3) CIC may be present in the circulation but the principle immune complex formation in the subepithelial space may occur *in situ* with "growth" of the deposits as more and more circulating antibody reacts with fixed antigen.

The controversy regarding the pathogenesis of AICN was initiated by the study of the passive model of membranous nephropathy produced by the administration of heterologous anti-Fx1A antiserum. Van Damme and co-workers (24) have shown early binding of this heterologous antibody to glomerular antigen. The antigen is associated with the subepithelial space and may be part of the epithelial cell membrane or the lamina rara externa of the glomerular basement membrane. It appears distinct from the glomerular basement membrane antigen responsible for induction of antibody operative in nephrotoxic serum nephritis. Only the 7 S, IgG antibody is necessary to transfer the disease. Couser and co-workers (5) have shown glomerular binding of heterologous antibody when injected in the isolated perfused rat kidney. These studies seem to eliminate the possibility of circu-

lating immune complex formation contributing to the deposited immunoglobulin. The passive heterologous model has not been evaluated utilizing antisera to purified RTE α_5 .

In the heterologous passive transfer model, the autologous phase and continued disease is dependent upon production of rat-antirabbit IgG which binds to glomerular bound rabbit-antirat Fx1A. Evaluation of this phase (25) established the binding of free antibody to a glomerular bound antigen as a mechanism which results in membranous nephropathy. In this respect, the heterologous passive transfer model is not directly analogous to either the homologous passive transfer or actively induced AICN models. It is quite possible that the heterologous antibody produced to the crude Fx1A has antibody specificity to an antigen that the glomerular epithelial and tubular epithelial cells share, and that this specificity accounts for the early binding of the passively administered antibody to the glomerulus and the target for the autologous phase. Several investigators have now attempted to duplicate the passive transfer experiments utilizing homologous antibody. Discrepant results have been obtained with homologous antiserum and eluted antibody evaluated *in vitro*, *in vivo*, with isolated glomeruli, and in the isolated perfused kidney (16,18,23). The controversy is yet to be resolved; however, it is possible that deposition of immune complexes from the circulation as well as *in situ* binding of antibody play a role in the development of AICN. Clarification of the precise pathogenesis of these models of membranous nephropathy and the relative contribution of the two likely mechanisms will require further evaluation.

Acknowledgments: The authors wish to thank Peggy Miles, David Strong, and Janine McVay for excellent technical assistance, Dr. John Hoyer for advice, and Kay Anderson and Patricia Shamblin for preparation of the manuscript.

Human thyroglobulin was kindly provided by Dr. Gildon Beall.

Date of acceptance: March 5, 1980. This paper was presented in part at the Annual Meeting of the American Society of Nephrology in Washington, D.C., November 1977.

This work was supported in part by a grant from the Kidney Foundation of Southern California; a General Research Support Grant provided by the National Institutes of Health, NIH SO7 RR 05551-14; and a gift from Arthur U. Rivin, M.D.

Doctor Abrass was supported as a Research Fellow of the National Kidney Foundation.

Address reprint requests to: Christine K. Abrass, M.D., Nephrology Section, VA Medical Center Sepulveda, 16111 Plummer Street, Sepulveda, California 91343.

REFERENCES

1. Barabas AZ, Nagi AH, Lannigan R: Induction of an autologous immune complex nephritis in rats by intravenous injection of heterologous anti-rat kidney tubular antibody. *Br J Exp Pathol* 55: 47, 1974
2. Border W, Abrass C, Hall C, Brown C, Glasscock R, Coggins C: Detection of circulating immune complexes (CIC) in adult idiopathic nephrotic syndrome (AINS) (abstr). *Kidney Int* 12:510, 1977
3. Border WA, Cohen AH: Role of immune complex charge in experimental glomerulonephritis (abstr). *Kidney Int* 16:796, 1979
4. Casali P, Bossus A, Carpentier NA, Lambert PH: Solid phase enzyme immunoassay or radioimmunoassay for the detection of immune complexes based on their recognition by conglutinin; con-

- glutinin binding test: a comparative study with ^{125}I -labeled C1q binding and Raji cell RIA tests. *Clin Exp Immunol* 29:342, 1977
5. Couser WG, Steinmuller DR, Stilmant MM, Salant DJ, Lowenstein LM: Experimental glomerulonephritis in the isolated perfused rat kidney. *J Clin Invest* 62:1275, 1978
 6. Edgington TS, Glasscock RJ, Dixon FJ: Autologous immune complex pathogenesis of experimental allergic glomerulonephritis. *Science* 155:1432, 1967
 7. Edgington TS, Glasscock RJ, Dixon FJ: Characterization and isolation of specific renal tubular epithelial antigens. *J Immunol* 99:1199, 1967
 8. Edgington TS, Glasscock RJ, Dixon FJ: Autologous immune complex nephritis induced with renal tubular antigen. I. Identification and isolation of the pathogenetic antigen. *J Exp Med* 127:555, 1968
 9. Feenstra K, Lee R, Greben HA, Arends A, Hoedemaeker PJ: Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. I. The natural history: a histologic and immunohistologic study at the light microscopic and ultrastructural level. *Lab Invest* 32:235, 1975
 10. Glasscock RJ, Edgington TS, Watson JI, Dixon FJ: Autologous immune complex nephritis induced with renal tubular antigen. II. The pathogenetic mechanism. *J Exp Med* 127:573, 1968
 11. Hay FC, Nineham LJ, Roitt IM: Routine assay for the detection of immune complexes of known immunoglobulin class using solid phase C1q. *Clin Exp Immunol* 24:396, 1976
 12. Heymann W, Hackel DB, Harwood S, Wilson SGF, Hunter JLP: Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspensions. *Proc Soc Exp Biol Med* 100:660, 1959
 13. Kingsbury FB, Clark CP: The rapid determination of albumin in urine. *J Lab Clin Med* 11:981, 1926
 14. Lambert PH, Dixon FJ, Fubler RH: A WHO collaborative study for the evaluation of eighteen methods for detecting immune complexes in serum. *J Clin Lab Immunol* 1:1, 1978
 15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265, 1951
 16. Makker SP, Mourthy B, Kirson I: On the hypothesis of in situ immune complex (ISIC) formation in Heymann nephritis (HN) of rats (abstr). *Kidney Int* 16:798, 1979
 17. McCahey PH, Dixon FJ: A method for trace iodination of proteins for immunologic studies. *Int Arch Allergy Appl Immunol* 29:185, 1966
 18. Neale TJ, Wilson CB: Fixed glomerular antigen in Heymann's nephritis: eluted antibody reactivity with normal rat glomeruli (abstr). *Kidney Int* 16:799, 1979
 19. Nydegger UE, Lambert PH, Gerber H, Miescher PA: Circulating immune complexes in the serum in systemic lupus erythematosus and in carriers of hepatitis B antigen: quantitation by binding to radiolabeled C1q. *J Clin Invest* 54:297, 1974
 20. Rossen RD, Reisberg MA, Singer DB, Schloeder FX, Suki WN, Hill LL, Eknayan G: Soluble immune complexes in sera of patients with nephritis. *Kidney Int* 10:256, 1976
 21. Schoenfeld LS, Glasscock RJ: Renal tubular antigen excretion in normal human urine. I. Immunochemical identification. *Kidney Int* 3:309, 1973
 22. Soltis RD, Morris MJ, Hasz DE, Wilson ID: The influence of endogenous serum C1q in the ^{125}I -C1q binding test. *J Lab Clin Med* 93:238, 1979
 23. Sugisaki T, Klassen J, Andres GA, Milgrom F, McCluskey RT: Passive transfer of Heymann nephritis with serum. *Kidney Int* 3:66, 1973
 24. Van Damme BJC, Fleuren GJ, Bakker WW, Vernier RL, Hoedemaeker PJ: Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. *Lab Invest* 38:502, 1978
 25. van Es LA, Blok APR, Schoenfeld L, Glasscock RJ: Chronic nephritis induced by antibodies reacting with glomerular-bound immune complexes. *Kidney Int* 11:106, 1977
 26. Weichselbaum TE: An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. *Am J Clin Pathol* 16:40, 1946
 27. Deleted in proof.
 28. Wilson CB, Dixon FJ: Antigen quantitation in experimental immune complex glomerulonephritis. I. Acute serum sickness. *J Immunol* 105:279, 1970
 29. Woodroffe AJ, Border WA, Theofilopoulos AN, Götze O, Glasscock RJ, Dixon FJ, Wilson CB: Detection of circulating immune complexes in patients with glomerulonephritis. *Kidney Int* 12:268, 1977
 30. Yonemasu K, Stroud RM: C1q: rapid purification method for preparation of monospecific antisera and for biochemical studies. *J Immunol* 106:304, 1971
 31. Zubler RH, Lange G, Lambert PH, Miescher PA: Detection of immune complexes in untreated sera by a modified ^{125}I C1q binding test. *J Immunol* 116:232, 1976

STIC-ILL

From: Romeo, David
Sent: Monday, June 04, 2001 11:44 AM
T : STIC-ILL
Subject: 09503421 Thiel S, Vorup-Jensen T, Stover CM, Schwaeble W, Laursen SB, Poulsen K,

APL
Q.12
MIC

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

Thiel S, Vorup-Jensen T, Stover CM, Schwaeble W, Laursen SB, Poulsen K,
Willis AC, Eggleton P, Hansen S, Holmskov U, Reid KB, Jensenius JC.

A second serine protease associated with mannan-binding lectin that activates complement.

Nature. 1997 Apr 3;386(6624):506-10.

PMID: 9087411

12. Gersten, R. E. *et al.* The thrombin receptor's specificity for agonist peptide is defined by its extracellular surface. *Nature* **368**, 648–651 (1994).
13. Amatruda, T. T., Steele, D. A., Slepak, V. Z. & Simon, M. I. Go19, a G protein α subunit specifically expressed in hematopoietic cells. *J. Biol. Chem.* **266**, 5587–5591 (1991).
14. Kettner, C. & Shaw, E. D. pht-pro-arg-CH₂Cl: a selective affinity label for thrombin. *Thrombosis Res.* **14**, 969–973 (1979).
15. Rydel, T. J. *et al.* Crystallographic structure of human γ -thrombin. *J. Biol. Chem.* **269**, 22000–22006 (1994).
16. Skrzypczak, J. E. *et al.* Structure of the hirugen and hirulog 1 complexes of α -thrombin. *J. Mol. Biol.* **221**, 1379–1393 (1991).
17. Scarborough, R. M. *et al.* Tethered ligand agonist peptides: structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J. Biol. Chem.* **267**, 13146–13149 (1992).
18. Hung, D. T., Vu, T. K. H., Wheaton, V. L., Ishii, K. & Coughlin, S. R. The cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. Blocking antiserum to the thrombin receptor's hirudin-like domain. *J. Clin. Invest.* **89**, 1350–1353 (1992).
19. Brass, L. F. *et al.* Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J. Biol. Chem.* **267**, 13753–13758 (1992).
20. Kinlough Rathbone, R., Perry, D. W., Guccione, M. A., Rand, M. L. & Packham, M. A. Degranulation of human platelets by the thrombin receptor peptide SFLLRN: comparison with J-aggregation by thrombin. *Thromb. Haemost.* **70**, 1019–1023 (1993).
21. Goodwin, C. A. *et al.* Thrombin receptor activating peptide does not stimulate platelet procoagulant activity. *Biochem. Biophys. Res. Commun.* **202**, 321–327 (1994).
22. Liu, L. F., Piumiglia, K., Cole, Y. P. & Feinstein, M. B. Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. *Biochem. J.* **303**, 391–400 (1994).
23. Kramer, R. M. *et al.* Differential activation of cytosolic phospholipase A2 (cPLA2) by thrombin and thrombin receptor agonist peptide in human platelets: evidence for activation of cPLA2 independent of the mitogen-activated protein kinases ERK1/2. *J. Biol. Chem.* **270**, 14816–14823 (1995).
24. Connolly, T. M. *et al.* Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thromb. Haemost.* **72**, 627–633 (1994).
25. Derian, C. K., Santulli, R. J., Tomko, K. A., Haertlein, B. J. & Andrade-Gordon, P. Species differences in platelet responses to thrombin and SFLLRN. Receptor-mediated calcium mobilization and aggregation and regulation by protein kinases. *Thrombosis Res.* **6**, 505–519 (1995).
26. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J. & Sealfon, S. C. Sequence alignment of the G-protein coupled receptor superfamily. *DNA and Cell* **11**, 1–20 (1992).
27. Ishii, K., Hein, L., Koblika, B. & Coughlin, S. R. Kinetics of thrombin receptor cleavage on intact cells: relation to signaling. *J. Biol. Chem.* **268**, 9780–9786 (1993).
28. Williams, J. A., McChesney, D. J., Calayag, M. C., Lingappa, V. R. & Logsdon, C. D. Expression of receptors for cholecystokinin and other Ca^{2+} -mobilizing hormones in *Xenopus* oocytes. *Proc. Natl Acad. Sci. USA* **85**, 4939–4943 (1988).
29. Nanevitz, T., Wang, L., Chen, M., Ishii, K. & Coughlin, S. R. Activating mutations in the thrombin receptor's agonist recognition domain. Mutations in a G-protein-coupled receptor's extracellular domain cause transmembrane signaling. *J. Biol. Chem.* **271**, 702–706 (1996).
30. Seifer, S. J., Peters, K. G., O'Keefe, J. & Coughlin, S. R. Disparate temporal expression of the prothrombin and thrombin receptor genes during mouse development. *Am. J. Pathol.* **144**, 60–69 (1993).

Acknowledgements. We thank C. Turck for peptide sequencing, J. Fenton II for α -thrombin and its derivatives, M. Simon for Go₁₄ cDNA, M. Davis for pBJ1, and H. Bourne for critical reading of this manuscript.

Correspondence and requests for materials should be addressed to S.R.C. (e-mail: shaun_coughlin.src@quickmail.ucsf.edu). Sequences of mouse and human cDNAs for PAR3 have been deposited in GenBank, accession numbers U92971 and U92972.

A second serine protease associated with mannan-binding lectin that activates complement

Steffen Thiel, Thomas Vorup-Jensen, Cordula M. Stover, Wilhelm Schneebli*, Steen B. Laursen, Knud Poulsen, Anthony C. Willis†, Paul Eggleston†, Søren Hansen, Uffe Holmskov†, Kenneth B. M. Reid† & Jens C. Jensenius

Department of Medical Microbiology and Immunology, University of Aarhus, DK 8000, Aarhus, Denmark

* Department of Microbiology and Immunology, University of Leicester, Leicester LE1 9HN, UK

† MRC Immunochemistry Unit, University of Oxford, Oxford OX1 3QU, UK

‡ Department of Medical Microbiology, University of Odense, Odense DK 5000, Denmark

The complement system comprises a complex array of enzymes and non-enzymatic proteins that is essential for the operation of the innate as well as the adaptive immune defence¹. The complement system can be activated in three ways: by the classical pathway which is initiated by antibody-antigen complexes, by

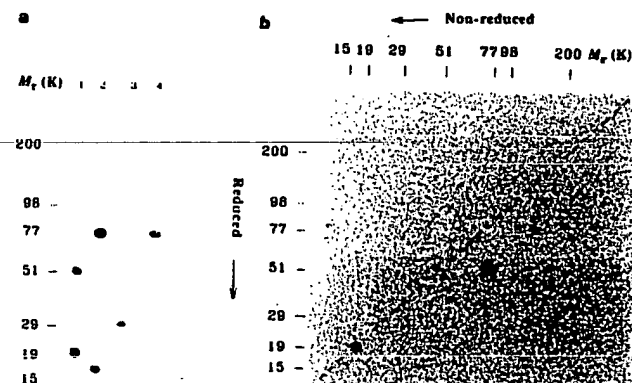


Figure 1 Western blotting of human plasma proteins purified by sugar affinity chromatography. One- and two-dimensional SDS-PAGE of this preparation was followed by blotting onto polyvinylidene difluoride (PVDF) membranes and development with various antibodies. **a**, One-dimensional electrophoresis; lanes 1 and 3 were run under reducing conditions and lanes 2 and 4 under non-reducing conditions. Lanes 1 and 2 were developed with anti-N'-MASP-2 antibody, lanes 3 and 4 with anti-C'-MASP-2 antibody. **b**, SDS-PAGE in two dimensions; the first dimension was run under non-reducing conditions. The lane was cut out, incubated in sample buffer containing DTT, placed on top of another SDS-polyacrylamide gel and electrophoresed; the gel was then blotted and the blot developed with anti-N'-MASP-2 antibody. The positions of M_r markers are indicated.

the alternative pathway initiated by certain structures on microbial surfaces, and by an antibody-independent pathway² that is initiated by the binding of mannan-binding lectin (MBL; first described as mannan-binding protein³) to carbohydrates. MBL is structurally related to the complement C1 subcomponent, C1q, and seems to activate the complement system through an associated serine protease known as MASP (ref. 4) or p100 (ref. 5), which is similar to C1r and C1s of the classical pathway. MBL binds to specific carbohydrate structures found on the surface of a range of microorganisms, including bacteria, yeasts, parasitic protozoa and viruses⁴, and exhibits antibacterial activity through killing mediated by the terminal, lytic complement components⁷ or by promoting phagocytosis⁸. The level of MBL in plasma is genetically determined^{9–11}, and deficiency is associated with frequent infections in childhood^{12,13}, and possibly also in adults^{14,15} (for review, see ref. 6). We have now identified a new MBL-associated serine protease (MASP-2) which shows a striking homology with the previously reported MASP (MASP-1) and the two C1q-associated serine proteases C1r and C1s. Thus complement activation through MBL, like the classical pathway, involves two serine proteases and may antedate the development of the specific immune system of vertebrates.

Human plasma proteins and protein complexes that bind to carbohydrates in a calcium-dependent manner (lectins and lectin-associated proteins) were purified by affinity chromatography on mannan- and N-acetylglucosamine-derivatized Sepharose beads. This protein preparation was analysed by SDS-PAGE and blotting onto a PVDF-membrane. Development of the blot with chicken antibody raised against a bovine lectin preparation¹⁶ revealed a protein of relative molecular mass 52,000 (M_r 52K), in addition to MBL at 32K. The 52K band was subjected to amino-terminal amino-acid sequence analysis. The sequence showed similarity to

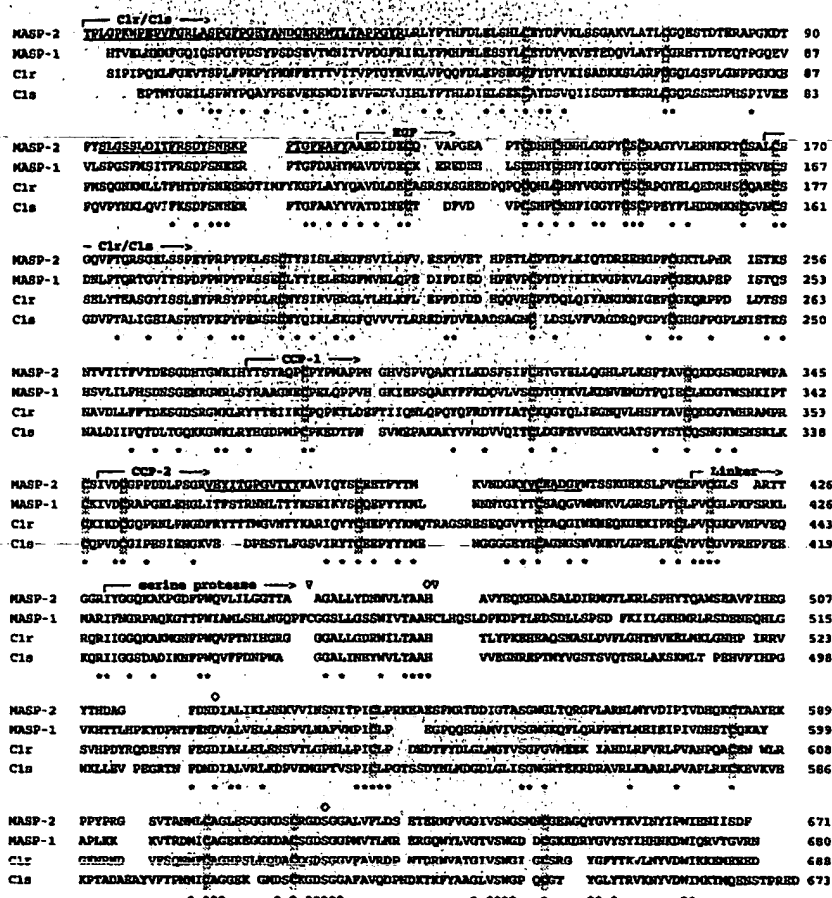


Figure 2 Sequence alignment of the amino-acid sequences of MASP-2 (clone phl-4), MASP-1 (refs 17, 22), C1r (refs 23, 24) and C1s (refs 25, 26) beginning with the N-terminal amino acid of the mature proteins (the putative leader sequence, MRLLTLGLLGGSVA, of MASP-2 is omitted). A bias of 6 was added to each term of the mutation data matrix (250PAMS) and a break penalty of 6 was used. Identical residues in all four species are indicated by asterisks. The beginning of the C1r/C1s-like domains, the EGF-like domain and the CCP domains are indicated above the sequences. Aligned cysteines are shaded. The potential cleavage site between Arg and Ile residues, which generates heavy and light chains, is identical to the site at which the serine protease domain starts. The three amino-acid residues that are essential for the active centre in serine proteases (His 468, Asp 517 and Ser 618), are indicated by diamonds. The cysteines in the histidine loop of MASP-1 are marked (inverted triangles). Sequences obtained from peptides are underlined.

that of the previously described MASP (MASP-1). Antibody raised against a synthetic peptide representing the 19 N-terminal amino acids (anti-N'MASP-2 antiserum) recognized the 52K molecule as well as a molecule with a mobility corresponding to 20K (Fig. 1, lane 1). Under non-reducing conditions, a polypeptide of 76K was detected with anti-N'MASP-2 antiserum (Fig. 1, lane 2), indicating the presence of interchain disulphide bonds. The 20K polypeptide had the same N-terminal sequence as the 52K polypeptide and is likely to represent a truncated form of it. The directly determined amino-acid sequences (N-terminal, as well as those of internal peptides) are indicated in Fig. 2. Two-dimensional SDS-PAGE, in which the first dimension was under non-reducing conditions and the second dimension under reducing conditions, showed the 52K polypeptide to be part of a disulphide-linked protein of M_r 76K. A polypeptide of 31K (Fig. 1, lane 3), probably representing the remainder of the protein, was also recognized as part of the 76K protein by an antiserum (anti-C'MASP-2) raised against synthetic peptides from the C-terminal sequence of the protein (determined by cDNA sequencing; see below). The 76K band seen with the anti-N'MASP-2 antibody under non-reducing conditions was also recognized by the anti-C'MASP-2 antibody (Fig. 1, lane 4).

The liver is the primary site of synthesis of C1r, C1s and MASP-1. Thus, RNA from liver was used as the template for polymerase chain reaction with reverse transcription (RT-PCR), with primers deduced from the peptide sequences. The nucleotide sequence of the resulting 300-base-pair (bp) RT-PCR product contained an open reading frame (ORF), with a deduced amino-acid sequence confirming the sequences of the peptides from which the primers were derived, as well as that of another of the sequenced peptides. The RT-PCR-derived product was used as a probe for screening a human liver cDNA library, revealing 16 hybridizing clones, of which the four longest (phl-1, 2, 3 and 4) were sequenced in full. Sequence

analysis revealed that all four clones represent reverse transcripts of the same novel human mRNA species. The longest clone, phl-4, comprises 2,475 bp starting with a 5' untranslated region of 36 bp, followed by an ORF of 2,061 bp and a 3' untranslated region of 378 bp, ending with a poly(A) tail. The nucleotide sequence of phl-4 is deposited at the EMBL nucleotide sequence data base (accession no. Y09926). Although the sequence of phl-1 and -2 were in total agreement with phl-4, the nucleotide sequence of phl-3 differs from phl-4 at two positions, a transversion at nucleotide position 1,147 (G to T) and a transition at position 1,515 (C to T). The first change leads to the replacement of Asp 356 with tyrosine. All clones were isolated from a liver library transcribed from RNA from a single donor, and the observed difference may represent polymorphism in the MASP-2 gene, or is due to an error created during construction of the library.

The amino-acid sequences of the N terminus as well as all sequenced peptides were identified in the sequence deduced from clone phl-4. The ORF encodes a polypeptide chain of 686 amino acids, including a signal peptide of 15 residues (Fig. 2). Omitting the signal peptide, the calculated M_r is 74,153, in agreement with the 76K observed on SDS-PAGE (Fig. 1), the isoelectric point is 5.43, and the molar extinction coefficient is 113,640 (absorbance at 280 nm of 1.54 at 1 mg ml⁻¹). In contrast to MASP-1, the sequence contains no sites for N-linked glycosylation. The three amino-acid residues that are essential for the active centre in serine proteases (His 468, Asp 517 and Ser 618) are present. The amino-acid sequence is homologous to that of MASP-1, C1r and C1s (Fig. 2). These four proteins have in common their domain organization, featuring one C1r/C1s-like domain, one epidermal-growth-factor-like (EGF-like) domain, followed by a second C1r/C1s-like domain, two complement control protein (CCP) domains, and a serine protease domain. The key residues involved in the calcium-binding

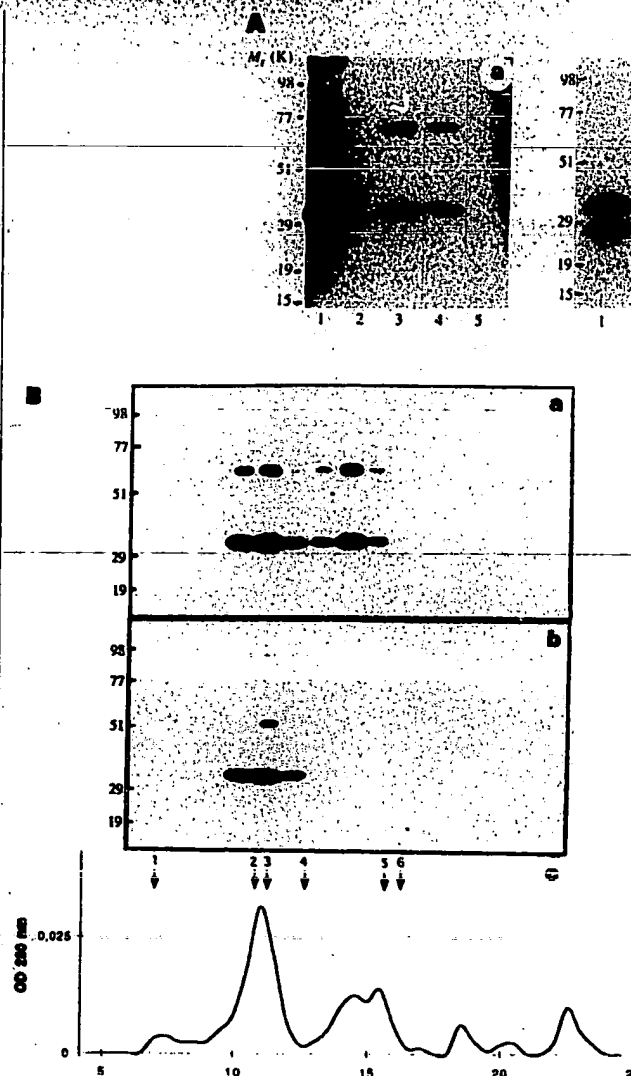


Figure 3 Molecular complex between MBL, MASP-1 and MASP-2. **A**, Affinity purification of MBL/MASP complexes. Microtitre wells were coated with monoclonal anti-MBL or control monoclonal murine IgG1, incubated with either one of two different lectin preparations (1 and 2), and the bound proteins were eluted and analysed by SDS-PAGE under reducing conditions and by western blotting. Blot **a** was developed with anti-MBL antibody, blot **b** with anti-C' MASP-1 antibody, and blot **c** with anti-N' MASP-2 antibody. Lane 1, unfractionated lectin, preparation (1); lanes 3 and 4, eluates from wells coated with anti-MBL antibody and incubated with lectin preparations (2) and (1), respectively; lanes 2 and 5, eluates from wells coated with normal IgG and incubated with lectin preparations (2) and (1), respectively. **B**, The lectin preparation was subjected to gel-permeation chromatography on a Superose-6 column in buffer containing calcium. **a**, Analysis of the fractions by western blotting using monoclonal anti-MBL antibody. The band at ~60K is seen in all MBL preparations and is recognized by all anti-MBL antibodies tested (monoclonal as well as polyclonal) and probably represents a non-reducible dimer of the 32K polypeptide chain. **b**, Same analysis, using anti-N' MASP-2 antibody (developing the upper band of 52K followed by anti-C' MASP-1 antibody (developing the lower band of 31K). For technical reasons, the 20K truncated MASP-2 is not seen here as the blot was partially stripped between incubations with anti-MASP-2 and anti-MASP-1. **c**, Numbered arrows on the chromatography elution profile indicate the void volume (1) and the elution positions for the marker proteins IgM (2), C1q (3), thyroglobulin (4), IgG (5) and serum albumin (6).

motif in the EGF-like domains are present in the sequence, as well as in MASP-1, C1r and C1s. In addition, the substrate specificity-related residue, six residues before the active-site serine, is aspartic acid in all four proteins. MASP-1, C1r and C1s are all activated by cleavage of the peptide bond between the residues arginine and isoleucine located between the second CCP domain and the serine protease domain. The resulting polypeptide chains (the larger is referred to as the 'heavy' chain and the smaller as 'light' chain) are held together by a disulphide bond. By analogy, our results indicate that the 52K polypeptide, recognized by antibody against the N-terminal of MASP-2 after SDS-PAGE under reducing conditions, is the heavy chain of MASP-2, whereas the 31K polypeptide, recognized by antibody against the C terminus of MASP-2, is the light chain. As seen in Fig. 2, arginine and isoleucine are present in MASP-2 at the expected positions between the second CCP domain and the protease domain.

Identities and similarities between the four proteins were studied from the sequence alignment shown in Fig. 2. Identity between the four proteins, all in the range of 39 to 45%, gives no clue as to functional relatedness. The similarity scores (taking into account residues of similar nature as well as identical residues) between the proteins are between 39 and 52%, with the least similarity being between MASP-1 and C1s (39%) and the highest similarity between MASP-1 and C1r (52%) and between MASP-1 and MASP-2 (52%). MASP-2 shows comparable similarity with C1r (46%) and C1s

(47%). The similarity score between C1s and MASP-2 is significantly higher than that between C1s and MASP-1, whereas MASP-1 is more similar to C1r than to C1s, suggesting that MASP-2, like C1s, could be the C2 and C4 cleaving enzyme. Several features of the sequences suggest that MASP-2, C1r and C1s have evolved by gene duplication and divergence from a MASP-1 ancestor. Only the MASP-1 sequence contains the histidine loop characteristic of trypsin-like serine proteases¹⁷. The active-site serine is encoded by a TCN codon (where N is A, T, G or C) in MASP-1 as in most serine proteases, whereas in MASP-2, C1r and C1s it is encoded by an AGY codon (where Y is T or C). In most serine proteases, including MASP-1, a proline residue is found at the third position downstream from the active-site serine, whereas a different amino acid is found in MASP-2, C1s and C1r (alanine in MASP-2 and C1s, valine in C1r). From these analogies, the catalytic domain of MASP-2 may be predicted to be encoded by a single exon as in C1r and C1s, whereas most other serine proteases, including MASP-1¹⁸, have split exons.

To investigate the association between MBL, MASP-1 and MASP-2, the lectin preparation was incubated in microtitre wells coated with monoclonal anti-MBL antibody, or, as a negative control, wells coated with nonspecific monoclonal immunoglobulin of the same subclass. The proteins captured by the antibody were eluted and analysed by SDS-PAGE and western blotting. The results (Fig. 3A) show that the anti-MBL antibody, in addition to binding MBL,

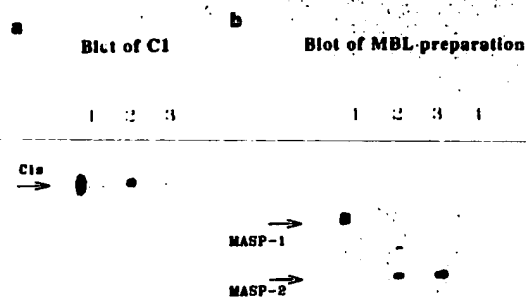


Figure 4 Activation of C4 by C1s and MASP-2 on western blots. **a**, Western blot of C1 separated under non-reducing conditions, without heating the sample before electrophoresis. Lane 1 was developed with anti-C1s antibody; lane 2 was incubated with human serum followed by anti-C4 antibody; lane 3 was as lane 2, except for the presence of serine-protease inhibitors during the incubation with serum. **b**, Western blot of an MBL preparation separated as in **a**. Lane 1 was developed with anti-N-MASP-1; lane 2 with anti-N-MASP-2; lane 3 was incubated with human serum at 37°C followed by anti-C4; in lane 4, the blot was preincubated with serine-protease inhibitors and the incubation with serum was also in the presence of inhibitors. MASP-1 shows a higher *M*, than MASP-2 owing to glycosylation¹⁷ and a polypeptide chain that is 9 amino acids longer.

captures both MASP-1 and MASP-2. Fractions from gel-permeation chromatography of the lectin preparation on Superose-6B CL were analysed for MBL, MASP-1 and MASP-2 (Fig. 3B). MBL was eluted in a main peak at a volume (*V*_e) corresponding to an *M*_r of 750K, with a smaller peak at 350K. MASP-1 and MASP-2 were found by western blotting to co-elute largely with the high-molecular-mass MBL. When the MBL preparation was chromatographed at pH 5, no MASP-1 or MASP-2 was associated with the MBL peak, as already reported for MASP-1 (ref. 19).

The classical complement activation pathway, like the MBL-initiated pathway, involves the generation of a C3-converting complex, C4b2b, through enzymatic activation of C4 and C2. In the C1 complex (C1q,r,s₂), this specific protease activity is exhibited by C1s after activation of this enzyme by C1r. When C4 is activated, its reactive thiol ester is exposed and C4b binds covalently to nearby amino or hydroxyl groups. The C4-activating abilities of MASP-1 and MASP-2 were compared. This was accomplished by separating an MBL/MASP preparation (prepared in the absence of enzyme inhibitors) by SDS-PAGE under non-reducing conditions, followed by blotting onto a PVDF membrane in the absence of SDS. The blot was examined for C4 converting activity by incubation with human serum at 37°C, followed by detection of deposited C4b with anti-C4 antibodies (Fig. 4). C4 was deposited at a position corresponding to the MASP-2 band, whereas no C4 deposition occurred at positions corresponding to MASP-1. MASP-1 was present in the activated state, as shown by SDS-PAGE under reducing conditions, where it appears as two bands at ~30K and 70K, respectively (results not shown). This C4 activation and deposition was inhibited by enzyme inhibitors (Fig. 4). No C4-stimulating activity could be detected when MBL/MASP was prepared in the presence of enzyme inhibitors added throughout the purification procedure. A preparation of C1 was similarly analysed, and C4 deposition, which could be inhibited by enzyme inhibitors, was observed at a position corresponding to C1r and C1s, which are not separated under our conditions.

Our results emphasize the similarity between complement activation through the MBL, or 'MBLectin' pathway of the innate immune system and the classical pathway of complement activation (Fig. 5). In both cases, the initiating molecular complexes are composed of an oligomeric ligand-binding component (MBL or

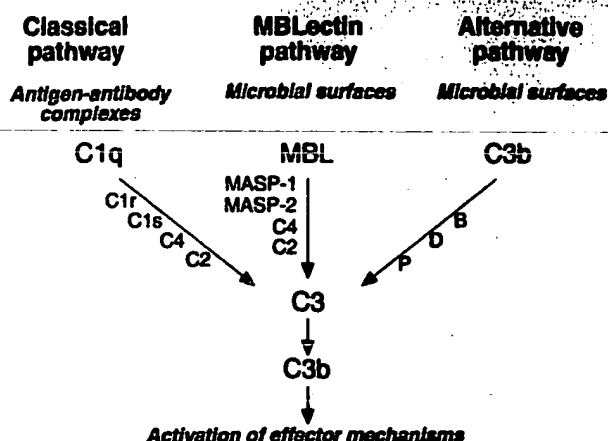


Figure 5 The three pathways of complement activation. Activation through the classical pathway is associated with the specific immune response found only in vertebrates, whereas the MBL, or 'MBLectin', pathway and the alternative pathway rely on innate recognition of foreign organisms and so probably predate the evolution of the specific immune system. All pathways converge on the activation of the central component C3 into C3b, which binds covalently to the microbial surface and mediates the effector functions of complement.

C1q, respectively) which, on reacting with ligands, activates the two associated serine proteases (MASP-1 and MASP-2 or C1r and C1s, respectively). Elucidation of the precise composition and stoichiometry of the MBL/MASP-1/MASP-2 complex, and the enzymatic properties of the two proteases, requires further investigation. □

Methods

Purification of plasma lectins and associated molecules. Pooled CPD plasma (2.5 l), diluted with buffer containing EDTA and enzyme inhibitors, was passed through Sepharose-2B CL and mannan-Sepharose. A thrombin inhibitor, PPACK (D-phenylalanyl-prolyl-arginyl-chloromethyl ketone), and CaCl₂ were added. The pool was passed through Sepharose-2B CL and mannan-Sepharose, and the proteins binding calcium-dependently to mannan-Sepharose were eluted with EDTA-containing buffer. The eluate was recalcified, passed through a GlcNAc-Sepharose column, which was eluted to yield 20 ml of lectin preparation.

Antibodies. Animals, primed with BCG (bacillus Calmette-Guérin vaccine), were immunized with synthetic peptides coupled to PPD (tuberculin-purified protein derivative) according to C. Koch (State Serum Institute, Copenhagen). Antibodies anti-N-MASP-1, anti-C-MASP-1 and anti-N-MASP-2 were from rabbits immunized with peptides corresponding to the first 19 amino-acid residues of MASP-1, the last 19 amino-acid residues of MASP-1, and the first 19 amino-acid residues of MASP-2, respectively. Chicken anti-C-MASP-2 antibody was from chickens immunized with a mixture of two peptides representing sequences in the C-terminal part of MASP-2 (residues 505–523 and 538–556). All peptides had an additional C-terminal cysteine for coupling. Antibody and normal chicken IgG was purified from yolk²⁰. Monoclonal anti-MBL antibody, IgG1-κ (clone 131-1) and control IgG1-κ (clone MOPC21) were purified by protein-A affinity chromatography. F(ab')₂ rabbit anti-human C4 and F(ab')₂ rabbit anti-human C1q were produced by pepsin digestion of rabbit anti-human C4 and rabbit anti-human C1q (Dako, Glostrup, Denmark). For staining of western blots, antibodies were used at 1 μg ml⁻¹. Bound chicken antibody was visualized with rabbit anti-chicken IgG followed by peroxidase-labelled goat anti-rabbit IgG and developed by enhanced chemiluminescence. Bound mouse and rabbit antibodies were visualized with peroxidase-labelled rabbit anti-mouse IgG and peroxidase-labelled goat anti-rabbit IgG, respectively.

Amino-acid sequencing of the 52K and the 20K polypeptides. The lectin preparation was concentrated, run on SDS-PAGE and transferred to a PVDF membrane. Two strips were developed with anti-bovine lectin antibody¹⁶. The

rest of the blot was stained with Coomassie brilliant blue. The band corresponding to the immunostained 52K band was cut out and sequenced on an Applied Biosystems protein sequencer. After production of anti-N' MASP-2 antibody, this was used on a similar western blot and the N termini of the proteins in the 52K and the 20K bands, developed with this antibody, were sequenced. Peptides were prepared by trypsin digestion of the proteins in the two bands from another blot, fractionated by reverse-phase chromatography and the peptides in the principal peaks were sequenced.

C4 activation on western blots. C1 was purified by incubating IgG-coupled Sepharose beads with human serum at 4°C. The beads were washed and incubated at 37°C for 30 min for activation of C1r and C1s. The beads were suspended in non-reducing sample buffer and, without boiling, run on SDS-PAGE, then blotted in the absence of SDS. A similar blot was made of an MBL preparation produced in the absence of enzyme inhibitors. Strips of the blots were incubated for 30 min at 37°C with 1:1% (v/v) human MBL-deficient serum, depleted of C1q by fractionation on Biorex-70. Blots were developed with biotinylated F(ab')₂ anti-C4 antibody, followed by peroxidase-labelled streptavidin and luminescence reagent. Parallel blots were treated with a serine-protease inhibitor (aminoethylbenzenesulphonyl fluoride), which was also present during incubation with serum. Other strips were directly developed with antibodies.

Cloning of the MASP-2 cDNA. First-strand synthesis of cDNA was carried out with 1.3 µg human liver RNA using a First-Strand cDNA synthesis kit (Pharmacia). PCR was done on this using degenerated sense and antisense primer derived from the amino-acid sequences EYANDQER and KPFTGFEEA, respectively. The PCR program consisted of 1 cycle of annealing at 50°C, 1 cycle of annealing at 55°C, and 33 cycles of annealing at 60°C. The resulting 300-bp PCR product was cloned into the *E. coli* plasmid pCRII using the TA-cloning kit (Invitrogen) and the nucleotide sequence of the insert was determined. The insert of this plasmid was used as the radioactively labelled probe for screening a total of 8 × 10⁵ clones in a commercial human liver library (Stratagene).

Received 30 September 1996; accepted 4 February 1997.

- Law, S. K. A. & Reid, K. B. M. *Complement* 2nd edn (ed. Male, D.) 1–88 (IRL, Oxford, 1996).
- Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T. & Yamashina, I. Serum lectin with known structure activates complement through the classical pathway. *J. Biol. Chem.* 262, 7451–7454 (1987).
- Kawasaki, T., Ettoh, R. & Yamashina, I. Isolation and characterization of a mannan-binding protein from rabbit liver. *Biochem. Biophys. Res. Commun.* 81, 1018–1204 (1978).
- Matsushita, M. & Fujita, T. Activation of the classical complement pathway by mannan-binding protein in association with a novel C1s-like serine protease. *J. Exp. Med.* 176, 1497–1502 (1992).
- Ji, Y.-H. et al. Activation of the C4 and C2 components of complement by a proteinase in serum bactericidal factor, Ra reactive factor. *J. Immunol.* 150, 571–578 (1993).
- Turner, M. W. Mannose-binding lectin: the pluripotent molecule of the innate immune system. *Immunol. Today* 17, 532–540 (1996).
- Kawasaki, N., Kawasaki, T. & Yamashina, I. A Serum lectin (mannan-binding protein) has complement-dependent bactericidal activity. *J. Biochem.* 106, 483–489 (1989).
- Kuhlman, M., Joiner, K. & Ezekowitz, R. A. B. The human mannan-binding protein functions as an opsonin. *J. Exp. Med.* 169, 1733–1745 (1989).
- Sumiya, M. et al. Molecular basis of opsonic defect in immunodeficient children. *Lancet* 337, 1569–1570 (1991).
- Lipscombe, R. J. et al. High frequencies in African and non-African populations of independent mutations in the mannan binding protein gene. *Hum. Mol. Genet.* 1, 709–715 (1992).
- Madsen, H. O. et al. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. *Immunogenetics* 40, 37–44 (1994).
- Super, M., Thiel, S., Lu, J., Levinsky, R. J. & Turner, M. W. Association of low levels of mannan-binding protein with a common defect of opsonisation. *Lancet* ii, 1236–1239 (1989).
- Garred, P., Madsen, H. O., Hofmann, B. & Svegaard, A. Increased frequency of homozygosity of abnormal mannan-binding protein alleles in patients with suspected immunodeficiency. *Lancet* 346, 941–943 (1995).
- Summerfield, J. A. et al. Mannose binding protein gene mutations associated with unusual and severe infections in adults. *Lancet* 345, 886–889 (1995).
- Nielsen, S. L., Andersen, P. L., Koch, C., Jensenius, J. C. & Thiel, S. The level of the serum opsonin, mannan-binding protein in HIV-1 antibody-positive patients. *Clin. Exp. Immunol.* 100, 219–222 (1995).
- Baastrop, G., Thiel, S., Isager, H., Svegaard, S. E. & Jensenius, J. C. Demonstration in human plasma of a lectin activity analogous to that of bovine conglutinin. *Scand. J. Immunol.* 26, 355–361 (1987).
- Sato, T., Endo, Y., Matsushita, M. & Fujita, T. Molecular characterization of a novel serine protease involved in activation of the complement system by mannan-binding protein. *Int. Immunol.* 6, 665–669 (1994).
- Endo, Y., Sato, T., Matsushita, M. & Fujita, T. Exon structure of the gene encoding the human mannan-binding protein-associated serine protease light chain: comparison with complement C1r and C1s genes. *Int. Immunol.* 9, 1355–1358 (1996).
- Tin, S. M. et al. Improvements on the purification of mannan-binding lectin and demonstration of its Ca²⁺-independent association with a C1s-like serine protease. *Biochem. J.* 319, 329–332 (1996).
- Jensenius, J. C., Andersen, P. L., Hsu, J., Crone, M. & Koch, C. Eggs: conveniently packaged antibodies. Methods for purification of yolk IgG. *J. Immunol. Meth.* 46, 63–66 (1981).
- Barton, G. I. Protein multiple sequence alignment and flexible pattern matching. *Meth. Enzymol.* 183, 403–428 (1990).
- Takada, F., Takayama, Y., Matsuse, H. & Kawakami, M. A new member of the C1s family of complement proteins found in a bactericidal factor, Ra-reactive factor, in human serum. *Biochem. Biophys. Res. Commun.* 196, 1003–1009 (1993).

- Journal, A. & Tsai, M. Cloning and sequencing of full-length cDNA encoding the precursor of human complement component C1r. *Biochem. J.* 240, 783–787 (1986).
- Lytus, S. P., Kurachi, K., Sakariassen, K. S. & Davie, E. W. Nucleotide sequence of cDNA coding for human complement C1r. *Biochemistry* 25, 4855–4863 (1986).
- Mackinnon, C. M., Carter, P. E., Smyth, S. J., Dunbar, B. & Fothergill, J. E. Molecular cloning of cDNA for human complement component C1s. The complete amino acid sequence. *Eur. J. Biochem.* 169, 547–553 (1987).
- Tsai, M., Duponchel, C., Meo, T. & Julier, C. Complete cDNA sequence of human complement C1S and close physical linkage of the homologous genes C1s and C1r. *Biochemistry* 26, 8516–8524 (1987).

Acknowledgements. We thank A. Day for alignment examination, and A. G. Hansen, J. Lovmand and J. Brøholm for technical assistance. The term 'MBLectin pathway' was suggested by C. Janeway. This investigation was supported by grants from the Danish MRC, the Danish National Research Foundation, the Novo Nordisk Foundation, the Alfred Benzon Foundation, the Plasmid Foundation, FEBS, the Wellcome Trust and the Deutsche Forschungsgemeinschaft.

Correspondence and requests for materials should be addressed to S.T. (e-mail: jensenius@svfcd.aau.dk).

Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue *in vivo*

H. E. Farrell, H. Vally, D. M. Lynch, P. Fleming, G. R. Shellam, A. A. Scalzo & N. J. Davis-Poynter

Department of Microbiology, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands 6907, Australia

Herpesviruses, such as murine and human cytomegalovirus (MCMV and HCMV), can establish a persistent infection within the host and have diverse mechanisms as protection from host immune defences¹. Several herpesvirus genes that are homologous to host immune modulators have been identified, and are implicated in viral evasion of the host immune response^{2,3}. The discovery of a viral major histocompatibility complex (MHC) class I homologue, encoded by HCMV⁴, led to speculation that it might function as an immune modulator and disrupt presentation of peptides by MHC class I to cytotoxic T cells⁵. However, there is no evidence concerning the biological significance of this gene during viral infection. Recent analysis of the MCMV genome has also demonstrated the presence of a MHC class I homologue⁶. Here we show that a recombinant MCMV, in which the gene encoding the class I homologue has been disrupted, has severely restricted replication during the acute stage of infection compared with wild-type MCMV. We demonstrate *in vivo* depletion studies that natural killer (NK) cells are responsible for the attenuated phenotype of the mutant. Thus the viral MHC class I homologue contributes to immune evasion through interference with NK cell-mediated clearance.

The sequence of the MCMV MHC class I homologue (designated m144) is remarkably similar to that of MHC class I proteins (Fig. 1). It is predicted to encode a 383 amino-acid type I membrane glycoprotein that bears sequence homology with the α1, α2 and α3 domains of MHC class I heavy chains^{7,8}. The highest level of sequence identity between m144 and MHC class I proteins is observed in the putative α3 domain (32% identity), including the cysteines known to form internal disulphide bonds in cellular class I proteins. Least conserved is the predicted α2 domain of m144 (20% identity), which is truncated compared to MHC class I proteins and the HCMV MHC class I homologue encoded by the UL18 gene. Low homology is also observed in the cytoplasmic region of m144, which is considerably longer than that of MHC class I proteins. Unlike UL18, which has 13 predicted N-linked glycosylation sites, m144 has only 4, which more closely approximates the number (1–3) found in HLA and H-2 class I molecules. UL18 and m144 are less conserved with each other than with MHC class I molecules from their respective species, suggesting that the genes were acquired independently or have diverged from an ancestral counterpart during adaptation of the viruses to their natural host.

STIC-ILL

MIC
BC583.C54
Adm

From: Romeo, David
Sent: Monday, June 04, 2001 1:28 PM
To: STIC-ILL
Subject: 09503421 Sturfelt G; Jonsson H; Hellmer G; Sjöholm A G

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Sturfelt G; Jonsson H; Hellmer G; Sjöholm A G
~~TI Clustering of neutrophil leucocytes in serum: possible role of~~
~~Clq-containing immune complexes.~~
SO CLINICAL AND EXPERIMENTAL IMMUNOLOGY, (1993 Aug) 93 (2) 237-41.
Journal code: DD7; 0057202. ISSN: 0009-9104.
AN 93351393

Clustering of neutrophil leucocytes in serum: possible role of C1q-containing immune complexes

G. STURFELT, H. JONSSON, G. HELLMER & A.G. SJÖHOLM* *Departments of Rheumatology and
Medical Microbiology, University Hospital of Lund, Lund, Sweden

(Accepted for publication 27 April 1993)

SUMMARY

Clustering activity for neutrophil granulocytes was generated in pooled normal human serum (NHS) by incubation of the serum with preformed IgG aggregates, but not in heat-treated NHS (56°C, 30 min), indicating that the function was complement-dependent. Judging from results of experiments with complement-deficient sera, and serum depleted of C1q, factor D and properdin, recruitment of the complement system beyond C1 was not required for induction of the activity. Zymosan treatment of NHS resulted in some neutrophil clustering activity, but recombinant C5a had a limited effect. C1q added to heat-treated NHS in conjunction with preformed IgG aggregates supported neutrophil clustering in a dose-dependent manner. The serum C1q inhibitor, a chondroitin 4-sulphate proteoglycan known to interact with the collagenous part of C1q, clearly reduced neutrophil clustering in heat-treated NHS supplemented with C1q and IgG aggregates. The C1q inhibitor also reduced the inherent neutrophil clustering activity of some sera from patients with systemic lupus erythematosus (SLE). Neutrophil clustering activity in SLE serum was earlier shown to be inversely related to the number of circulating neutrophils *in vivo*. Although the precise mechanisms remain unclear, we propose that C1q-containing immunoglobulin complexes mediate neutrophil clustering through C1q receptors, and that this might contribute to pathogenesis of immune complex diseases such as SLE.

Keywords neutrophil granulocytes neutrophil aggregation complement component C1q immune complexes systemic lupus erythematosus

INTRODUCTION

Intravascular formation of neutrophil granulocyte aggregates is thought to be of pathogenetic significance in systemic lupus erythematosus (SLE), particularly with regard to development of central nervous system and pulmonary disease manifestations [1-3].

We have described a simple assay for *in vitro* assessment of neutrophil clustering or aggregation in serum [4]. Sera from patients with severe SLE gave increased clustering of neutrophils from healthy donors. The clustering activity varied with the clinical course of the disease and was inversely related to the number of circulating neutrophils in the patients. C1q-binding immune complexes and neutrophil clustering activity were correlated, and clustering activity could be generated in normal serum with preformed IgG aggregates, indicating that immune complexes in conjunction with complement could be responsible.

The purpose of the present study was to identify possible complement-related ligands for induction of neutrophil cluster-

ing in human serum with preformed IgG aggregates. C1 subcomponents together with C4 and C3 proteins are known to be fixed to complement-activating immunoglobulin complexes [5], and contribution of C5-derived peptides generated in the course of complement activation [6] was also considered. The results suggested that C1q-containing immunoglobulin complexes were critically involved, and that further recruitment of the complement system was not required in the reaction.

MATERIALS AND METHODS

Human sera

Sera were frozen in aliquots at -70°C within 4 h after blood sampling. Pooled normal human serum (NHS) from 20 healthy hospital staff members was used as a reference in all experiments. Sera from persons with homozygous deficiency of C1q, C4, C2, C5 or C7 were available in the laboratory. For some experiments, normal serum from one donor was depleted of C1q, factor D and properdin as described by Sjöholm *et al.* [7]. Where indicated, NHS was inactivated by heat treatment (56°C, 30 min). Zymosan treatment of serum was performed as described previously [8]. Sera with high granulocyte clustering

Correspondence: G. Sturfelt, MD, PhD, Department of Rheumatology, University Hospital, S-22185 Lund, Sweden.

activity from five patients with definite SLE [4] were also investigated. The patients fulfilled four or more of the ACR classification criteria for SLE [9]. Disease activity was estimated by SLEDAI score [10].

Complement components

C1q was purified according to Tenner *et al.* [11]. To remove possible aggregates, C1q preparations were consistently subjected to gel filtration on a TSK-4000 column (LKB, Bromma, Sweden) in an FPLC system (Pharmacia, Uppsala, Sweden). After gel filtration, C1q preparations were used within 2 days. Factor D and properdin were purified according to published methods [12,13]. Concentrations of the purified complement proteins were determined by electroimmunoassay, assuming a reference normal serum pool to contain C1q at 70 mg/l, properdin at 25 mg/l and factor D at 1 mg/l [6]. C1q inhibitor, a chondroitin-4 sulphate proteoglycan, was purified from 1.5 ml of outdated ACD plasma essentially as described by Silvestri *et al.* [14]. In brief, C1q inhibitor in complex with C1q was isolated from plasma by repeated euglobulin precipitation, and was then separated from C1q by chromatography on a 1.6 × 26 cm column of concanavalin A (Con A) Sepharose (Pharmacia) to which C1q was quantitatively bound [14]. The C1q inhibitor recovered in the effluent was dialysed overnight against 20 volumes of Tris 0.02 mol/l, NaCl 0.2 mol/l, pH 7.5, and was applied to a column of DEAE-Sepharose (Pharmacia) equilibrated in the same buffer. The inhibitor was eluted with a sharp NaCl gradient to 1 mol/l. C1q inhibitory activity was detected by haemolytic assay [15] and by single diffusion precipitation of purified C1q in agarose [14]. The sulphated glycosaminoglycan content of the C1q inhibitor preparation (0.92 mg/ml, total yield 2.8 mg) was kindly determined by Dr S. Björnsson (Lund, Sweden) according to a recently described procedure [16].

Other reagents

Commercial polyclonal IgG (Kabi, Stockholm, Sweden) at 10 g/l was aggregated by heat (63°C, 30 min). After treatment, the heat-aggregated IgG (HAIG) was centrifuged at 2000 *g* for 30 min, the supernatant being used for addition to serum. Recombinant human C5a was purchased from Sigma Chemical Co. (St Louis, MO).

Assay for measurement of neutrophil clustering

The assay for *in vitro* measurement of neutrophil clustering has been described in detail [4]. In short, neutrophils (0.01 ml, 10⁷ neutrophils/ml) from healthy donors were added to 0.04 ml of test serum, incubated at room temperature for 1 min, whereafter single and clustered cells were counted within a standardized area. In each of duplicate preparations 200 single cells were counted. Clustering activity was expressed as the number of clustered cells in per cent of the total number of counted cells.

To generate neutrophil clustering activity, the sera were incubated for 60 min at 37°C with HAIG at a final concentration of 1 g/l. In reconstitution experiments, purified complement proteins were first added to serum depleted of C1q, factor D and properdin, or to heat-treated NHS at physiological concentrations. Sera from patients with SLE were used without pretreatment with HAIG. C1q inhibitor at specified concentrations was added to HAIG-treated sera or to SLE sera, followed by incubation at 37°C for 10 min before the addition of cells.

Table 1. Neutrophil clustering (%) in normal and complement-deficient sera with and without treatment with heat-aggregated IgG (HAIG)

	Untreated serum	Serum treated with HAIG
Normal serum	5	26
C2-deficient serum		
I	7	36
II	10	35
III	12	54
C4-deficient serum	ND	51
Hereditary angio-oedema serum	12	26
Properdin-deficient serum	ND	32
C3-deficient serum*	10	32
C5-deficient serum	3	31
C7-deficient serum		
I	13	42
II	5	38
III	9	28
C8-deficient serum	25	43

* Acquired C3 deficiency in a patient with C3 nephritic factor.

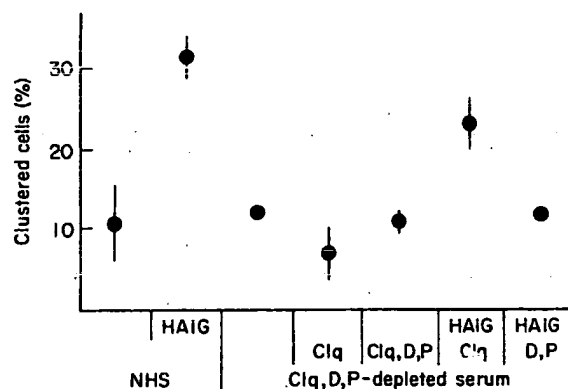


Fig. 1. Induction of neutrophil clustering with heat-aggregated IgG (HAIG) at 1 g/l in pooled normal human serum (NHS) and in normal serum depleted of C1q, factor D and properdin (C1q,D,P-depleted serum). Purified complement proteins were added to the depleted serum at physiological concentrations as indicated below the figure. Results of two separate experiments are shown. Neutrophil clustering activity is given as mean value and range.

RESULTS

Neutrophil clustering in complement-deficient and complement-depleted sera

In NHS the proportion of clustered cells varied between 5% and 25% of the total number of counted cells. In NHS treated with HAIG the number of clustered cells was consistently increased, and varied between 21% and 48%.

Addition of HAIG clearly promoted neutrophil clustering in sera from persons with homozygous deficiencies of C2, C5, C7 or C8 (Table 1). Similar findings were made in serum from a patient with hereditary angio-oedema and in serum from a

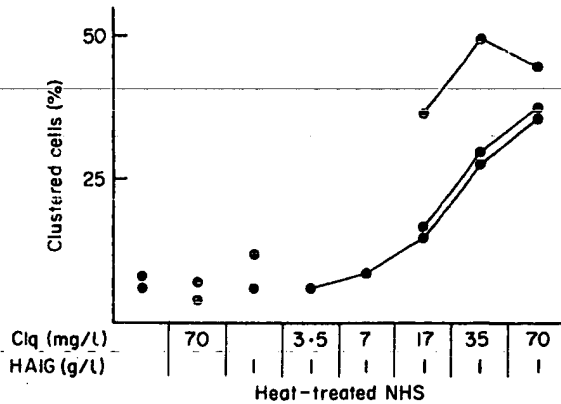


Fig. 2. Clq-dependent induction of neutrophil clustering activity in heat-treated (56°C, 30 min) pooled normal human serum (NHS). Clq and heat-aggregated IgG (HAIG) were added at the concentrations indicated below the figure. Results of three separate experiments are shown.

patient with acquired C3 deficiency. In addition, high neutrophil clustering activity was found in C4-deficient serum and properdin-deficient serum treated with HAIG.

Further experiments were carried out with normal serum artificially depleted of Clq, factor D and properdin (Fig. 1). The depleted serum did not support enhanced neutrophil clustering activity in the presence of HAIG. However, reconstitution with Clq, but not with factor D and properdin before addition of HAIG, produced a marked response with regard to neutrophil-clustering activity.

The results of experiments with complement-deficient and complement-depleted sera suggested that neutrophil clustering induced with HAIG required Clq or the C1 complex, and that further recruitment of the complement system was not necessary. In particular, the findings with C5-deficient serum argued against an important role of C5-derived peptides (C5a and C5a_{des arg}) in the assay. Zymosan-treated serum, known to be a

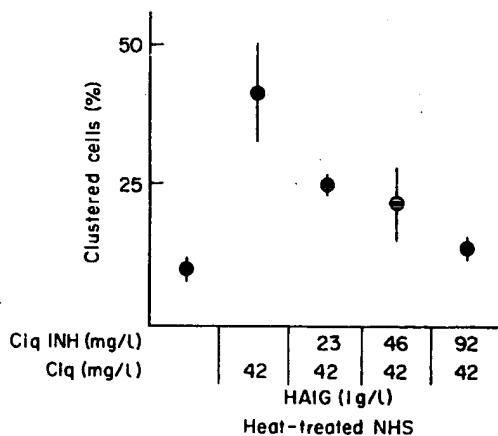


Fig. 3. Influence of Clq inhibitor (Clq INH) on neutrophil clustering in heat-treated (56°C, 30 min) pooled normal human serum (NHS) after incubation of the serum with Clq and heat-aggregated IgG (HAIG). Final concentrations of Clq INH, Clq and HAIG are given below the figure. Results of two separate experiments are shown. Neutrophil clustering activity is given as mean value and range.

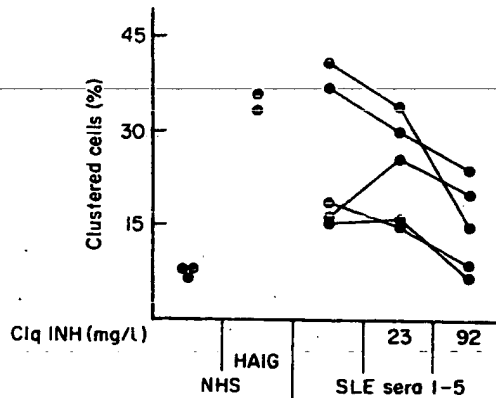


Fig. 4. Influence of Clq inhibitor (Clq INH) on neutrophil clustering in sera from five patients with active systemic lupus erythematosus (SLE) (SLE-sera 1-5). Clq-INH was added at the concentrations indicated below the figure. Results of included control experiments with pooled normal human serum (NHS) and heat-aggregated IgG (HAIG, 1 g/l) are shown to the left.

reliable source of C5-derived peptides [8], produced slightly less neutrophil clustering than normal serum activated with HAIG. Addition of recombinant C5a at a final concentration of 1-10 mg/l to normal serum resulted in very little neutrophil-clustering activity.

Neutrophil-clustering activity in heat-treated serum: effect of purified Clq

Background clustering in heat-treated NHS was similar to or less than that observed in NHS. Addition of HAIG to heat-treated NHS did not increase neutrophil clustering, nor did addition of Clq alone. When heat-treated NHS was reconstituted with Clq before addition of HAIG, Clq produced a dose-dependent increase of neutrophil-clustering activity in the serum (Fig. 2).

Inhibition experiments

In heat-treated NHS with Clq and aggregated IgG added, purified Clq inhibitor reduced neutrophil clustering in a dose-dependent manner (Fig. 3). Inhibitory doses were in the range 23-92 mg/l. The normal concentration of Clq inhibitor in serum is not known, but could be in the order of 20 mg/l assuming a 10% yield during the preparation procedure. This implies that the inhibitory doses were well beyond the normal concentration of Clq inhibitor.

Serum from five patients with active disease (SLEDAI scores 10-24) caused clustering from 17 to 43% of the total cell count. The morphology of cell clusters induced by SLE serum and by HAIG-activated serum was similar. In four of the five SLE sera, Clq inhibitor produced dose-dependent reduction of neutrophil clustering (Fig. 4).

DISCUSSION

Intravascular aggregation of neutrophil granulocytes is considered an important event in the inflammatory response, by promoting neutrophil contact for endothelial adhesion before transendothelial migration and accumulation of neutrophils at

extravascular sites [17]. Neutrophil expression of complement receptor type 3 (CR3), a member of the CD18 complex [18], appears to be critical for neutrophil aggregation and for adhesion to endothelium [19,20]. CR3-dependent neutrophil aggregation involves a cell surface counter-structure [21] that may be distinct from ICAM-1 and ICAM-2 [18]. Aggregation of neutrophils also requires cell activation as triggered by chemoattractants such as C5-derived peptides or other agents [19,21]. *In vivo*, intravascular chemoattractants and systemic complement activation induce neutropenia and neutrophil sequestration due to adherence of neutrophils to endothelium in the microvasculature [22,23].

Complement activation has been implied in neutrophil-mediated pathogenetic events associated with haemodialysis [24], the adult respiratory distress syndrome [25], and the 'post-perfusion syndrome' [26]. These studies have been focused on effects of C5-derived peptides. In the adult respiratory distress syndrome, neutrophil aggregating activity in plasma as ascribed to C5a was correlated with development of symptoms [25].

Sera from patients with SLE or Felty's syndrome have been reported to produce aggregation adherence to endothelium and superoxide generation of neutrophils *in vitro* [2]. Attempts to identify the serum factor(s) involved have been inconclusive [2-4].

In the present investigation, neutrophil aggregating activity in serum was assessed with a simple neutrophil clustering assay [4], whereas turbidimetry measurements have been employed in other studies [2,3,24,25]. Thus, findings might not be strictly comparable. Changes in light scattering are influenced by cell polarization and modification of subcellular structures [27]. Furthermore, cytochalasin B, an agent that enhances neutrophil aggregation responses [28] by interaction with actin [29], was used in the study of Abramson *et al.* [3]. In our assay, preformed IgG aggregates readily generated neutrophil clustering activity in normal serum, while no such effect was obtained in heat-treated serum. This argued against a role of cell activation mediated through FcγRII and FcγRIII [30], and clearly suggested a requirement for complement. It is questionable if the clustering effect of Zymosan-treated serum was due to C5-derived peptides, since recombinant C5a showed very limited activity.

The principal finding was that recruitment of the complement system beyond C1 was not necessary for efficient induction of neutrophil clustering. However, preformed IgG aggregates in combination with C1q produced marked activity, even in heat-treated serum. We conclude that the reaction was triggered by C1q-containing IgG complexes through interaction with neutrophil surface receptors for C1q [31].

C1q receptors recognize the collagenous part of C1q [32,33], and collagen structures of C-type lectins that resemble C1q [34]. The receptor binding site of C1q is masked by C1r and C1s in the C1q(C1r-C1s)₂ complex [32], but disassembly of the molecule by C1 inhibitor in conjunction with activation [35] exposes the ligand structure of fixed C1q. Like C1q receptors, the C1q inhibitor in serum [14] binds to the collagenous part of C1q [15]. C1q inhibitor virtually abolished neutrophil clustering elicited with C1q-containing complexes and the inherent neutrophil clustering activity of some SLE sera, indicating that the collagenous part of C1q was a significant ligand in the reaction.

To our knowledge, neutrophil clustering by a C1q-dependent mechanism has not been previously described. The pre-

vious finding of inverse correlation between neutrophil clustering activity and the number of circulating neutrophils in SLE [4] suggests that the activity is not a biologically inconsequential phenomenon. C1q can activate neutrophils as expressed by an oxidative response [36] with recruitment of partly unique signal transduction pathways [37]. It remains to be established if C1q-triggered neutrophil clustering resembles neutrophil aggregation induced by other stimuli in the requirement for continuous cell activation and CD18 leucocyte adhesion molecules [21], or if other mechanisms are involved.

The ligand requirements for induction of neutrophil clustering activity in serum strongly suggested involvement of C1q receptors, even if the precise mechanisms remain unclear. As proposed for platelets [38] and endothelial cells [39], C1q receptors might contribute to localization of C1q-containing immune complexes to neutrophil surfaces. Assuming that this leads to neutrophil clustering and enhanced margination of the cells, the findings relate to previous observations in disease conditions such as SLE [1-4]. Interestingly, C1 dissociation with possible exposure of immune complex-bound C1q is pronounced in active SLE [40,41]. We propose that C1q bound to immune complexes could be an important ligand for interaction with neutrophils in SLE, and perhaps also in other immune complex diseases.

ACKNOWLEDGMENTS

The study was supported by the Swedish Medical Research Council (B93-27X-09528-03B and B93-161-07921-07A), the National Board for Industrial and Technical Development, the Medical Faculty of the University of Lund, the Swedish National Association against Rheumatism, King Gustaf V's 80th Birthday Fund, Greta och Johan Rock's Foundation, Alfred Österlund's Foundation, Anna-Greta Crafoord's Foundation, Crafoord's Foundation, Nanna Svartz' Fund, Thelma Zoega's Fund, and the Foundation for Aid to the Disabled in Scania.

REFERENCES

- 1 Churg A, Franklin W, Chan KL, Kopp E, Carrington CB. Pulmonary hemorrhage and immune-complex deposition in the lung: complications in a patient with systemic lupus erythematosus. *Arch Pathol Lab Med* 1980; 104:388-91.
- 2 Hashimoto Y, Ziff M, Hued E. Increased endothelial cell adherence, aggregation and superoxide generation by neutrophils incubated in systemic lupus erythematosus and Felty's syndrome sera. *Arthritis Rheum* 1982; 25:1409-18.
- 3 Abramson SB, Given WP, Edelson HS, Weissman G. Neutrophil aggregation induced by sera from patients with active systemic lupus erythematosus. *Arthritis Rheum* 1983; 26:630-6.
- 4 Jonsson H, Sturfelt G. A novel assay for neutrophil clustering activity of human sera: relation to disease activity and neutropenia in systemic lupus erythematosus. *Ann Rheum Dis* 1990; 49:46-50.
- 5 Schifferli JA, Ng YC. The role of complement in the processing of immune complexes. *Baillieres Clin Immunol Allergy* 1988; 2:319-34.
- 6 Cooper NR. Laboratory investigation of complement proteins and complement receptors. *Baillieres Clin Immunol Allergy* 1988; 2:263-93.
- 7 Sjöholm AG, Selander B, Östenson S, Holmström E, Söderström C. Normal human serum depleted of C1q, factor D and properdin: its use in studies of complement activation. *APMIS* 1991; 99:1120-8.
- 8 Sturfelt G, Linder C, Sjöholm AG, Svensson B. Impaired monocyte motility in sera from patients with systemic lupus erythematosus. *Acta Path Microbiol Immunol Scand (C)* 1983; 91:151-8.

- 9 Tan EM, Cohen AS, Fries JF *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**:1271-7.
- 10 Bombardier C, Gladman DD, Urowitz MB, Chang CH, Charron D. Development and validation of the SLEDAI: an index of disease activity in SLE. *Arthritis Rheum* 1992; **35**:630-40.
- 11 Tenner AJ, Lesavre P, Cooper NR. Purification and radiolabeling of human C1q. *J Immunol* 1981; **127**:648-53.
- 12 Truedsson L, Sturfelt G. Human factor D of the alternative pathway: purification and quantitation by enzyme amplified electroimmunoassay. *J Immunol Methods* 1983; **63**:207-14.
- 13 Medicus RG, Esser AF, Fernandez HN, Muller-Eberhard HJ. Native and activated properdin: interconvertibility and identity of amino- and carboxy-terminal sequences. *J Immunol* 1980; **124**:602-6.
- 14 Silvestri L, Baker JR, Rodén L, Stroud RM. The C1q inhibitor in serum is a chondroitin 4-sulphate proteoglycan. *J Biol Chem* 1981; **256**:7383-7.
- 15 Ghebrehwet B. C1q inhibitor (C1qINH): functional properties and possible relationship to a lymphocyte membrane-associated C1q precipitin. *J Immunol* 1981; **126**:1837-42.
- 16 Björnsson S. Simultaneous preparation and quantitation of proteoglycans by precipitation with Alcian Blue. *Anal Biochem* 1993; **210**:282-91.
- 17 Harlan JM. Leukocyte-endothelial interactions. *Blood* 1985; **65**:513-25.
- 18 Arnaout MA. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* 1990; **75**:1037-50.
- 19 Schwartz BR, Ochs HD, Beatty PG, Harlan JM. A monoclonal antibody-defined membrane antigen complex is required for neutrophil-neutrophil aggregation. *Blood* 1985; **65**:1553-6.
- 20 Tonnesen MG, Anderson DC, Springer TA, Knedler A, Avdi N, Henson PM. Adherence of neutrophils to cultured human microvascular endothelial cells: Stimulation by chemotactic peptides and lipid mediators and dependence upon the Mac-1, LFA-1, p150, 95 glycoprotein family. *J Clin Invest* 1989; **83**:637-46.
- 21 Kuypers TW, Koenderman L, Weening RS, Verhoeven AJ, Roos D. Continuous cell activation is necessary for stable interaction of complement receptor type 3 with its counter-structure in the aggregation response of human neutrophils. *Eur J Immunol* 1990; **20**:501-8.
- 22 McCall CE, DeChatelet LR, Brown D, Lachmann P. New biological activity following intravascular activation of the complement cascade. *Nature* 1974; **249**:841-3.
- 23 Fehr J, Jacob HS. *In vitro* granulocyte adherence and *in vivo* margination. Two associated complement-dependent functions. *J Exp Med* 1977; **146**:641-52.
- 24 Craddock PR, Hammerschmidt D, White JG, Dalmaso AP, Jacob HS. Complement (C5a)-induced granulocyte aggregation *in vitro*: a possible mechanism of complement mediated leukostasis and leukopenia. *J Clin Invest* 1977; **60**:260-4.
- 25 Hammerschmidt D, Weaver L, Hudson L, Craddock PR, Jacob HS. Association of complement activation and elevated plasma C5a with adult respiratory distress syndrome. *Lancet* 1980; **i**:947-9.
- 26 Chenoweth DE, Cooper SW, Hughli TE, Stewart RW, Blackstone EH, Kirklin JW. Complement activation during pulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins. *N Engl J Med* 1981; **304**:497-502.
- 27 Yuli I, Snyderman R. Light scattering by polymorphonuclear leukocytes stimulated to aggregate under various pharmacologic conditions. *Blood* 1984; **64**:649-55.
- 28 Kaplan HB, Edelson HS, Friedman R, Weissman G. The roles of degranulation and superoxide anion generation in neutrophil aggregation. *Biochim Biophys Acta* 1982; **721**:55-63.
- 29 Ohmori H, Toyama S, Toyama S. Direct proof that the primary site of action of cytochalasin on cell motility processes is actin. *J Cell Biol* 1992; **116**:933-41.
- 30 Crockett-Torabi E, Fantone JC. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fc γ receptor-specific mechanisms. *J Immunol* 1990; **145**:3026-32.
- 31 Ghebrehwet B. Functions associated with the C1q receptor. *Behring Inst Mitt* 1989; **84**:204-15.
- 32 Tenner AJ, Cooper NR. Analysis of receptor-mediated C1q binding to human peripheral blood mononuclear cells. *J Immunol* 1980; **125**:1658-63.
- 33 Arvieux J, Reboul A, Bensa JC, Colomb MG. Characterization of the C1q receptor on a human macrophage cell line, U937. *Biochem J* 1984; **218**:547-55.
- 34 Malhotra R, Thiel S, Reid KBM, Sim RB. Human leukocyte C1q receptor binds other soluble proteins with collagen domains. *J Exp Med* 1990; **172**:955-9.
- 35 Laurell A-B, Mårtensson U, Sjöholm AG. C1 dissociation. Spontaneous generation in human serum of a trimer complex containing C1 inactivator, activated C1r, and zymogen C1s. *J Immunol* 1987; **139**:4145-51.
- 36 Tenner AJ, Cooper NR. Stimulation of a human polymorphonuclear leukocyte oxidative response by the C1q subunit of the first complement component. *J Immunol* 1982; **128**:2547-52.
- 37 Goodman EB, Tenner AJ. Signal transduction mechanisms of C1q-mediated superoxide production. Evidence for the involvement of temporally distinct staurosporine-insensitive and -sensitive pathways. *J Immunol* 1992; **148**:3920-8.
- 38 Peerschke EIB, Ghebrehwet B. Platelet interactions with C1q in whole blood and in the presence of immune complexes or aggregated IgG. *Clin Immunol Immunopathol* 1992; **63**:45-50.
- 39 Daha MR, Miltenburg AMM, Hiemstra PS, Klar-Mohamed N, Van Es LA, Van Hinsbergh VWM. The complement subcomponent C1q mediates binding of immune complexes and aggregates to endothelial cells *in vitro*. *Eur J Immunol* 1988; **18**:783-7.
- 40 Sturfelt G, Johnson U, Sjöholm AG. Sequential studies of complement activation in systemic lupus erythematosus. *Scand J Rheumatol* 1985; **14**:184-96.
- 41 Jonsson H, Sjöholm AG, Mårtensson U, Laurell A-B, Sturfelt G. C1 subcomponent complexes and C2 cleavage in active systemic lupus erythematosus. *Complement Inflamm* 1991; **8**:1-12.

STIC-ILL

RC583 C5
Adams

From: Romeo, David
Sent: Monday, June 04, 2001 12:52 PM
To: STIC-ILL
Subject: 09503421 Peterson K L; Zhang W; Lu P D; Keilbaugh S A; Peerschke E I; Ghebrehiwet

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Peterson K L; Zhang W; Lu P D; Keilbaugh S A; Peerschke E I; Ghebrehiwet B
TI The Clq-binding cell membrane proteins cClq-R and gClq-R are released from
activated cells: subcellular distribution and immunochemical
characterization.
SO CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY, (1997 Jul) 84 (1) 17-26.
Journal code: DEA; 0356637. ISSN: 0090-1229.
AN 97335214

The C1q-Binding Cell Membrane Proteins cC1q-R and gC1q-R Are Released from Activated Cells: Subcellular Distribution and Immunochemical Characterization¹

Keri L. Peterson,* Weibing Zhang,* Phoebe D. Lu,* Sue Ann Keilbaugh,*
Ellenor I. B. Peerschke,† and Berhane Ghebrehiwet*†

*Department of Medicine and †Department of Pathology, State University of New York, Stony Brook, New York 11794-8161

Two types of widely coexpressed cell surface C1q-binding proteins (C1q-R): a 60-kDa calreticulin-homolog which binds to the collagen-like "stalk" of C1q and a 33-kDa protein with affinity for the globular "heads" of the molecule, have been described. In this report, we show that the two molecules are also secreted by Raji cells and peripheral blood lymphocytes and can be isolated in soluble form from serum-free culture supernatant by HPLC purification using a Mono-Q column. The two purified soluble proteins had immunochemical and physical characteristics similar to their membrane counterparts in that both bound to intact C1q and to their respective C1q ligands, cC1q and gC1q. In addition, N-terminal amino acid sequence analyses of the soluble cC1q-R and gC1q-R were found to be identical to the reported sequences of the respective membrane-isolated proteins. Ligand blot analyses using biotinylated membrane or soluble cC1q-R and gC1q-R showed that both bind to the denatured and nondenatured A-chain and moderately to the C-chain of C1q. Moreover, like their membrane counterparts, the soluble proteins were found to inhibit serum C1q hemolytic activity. Although cC1q-R was released when both peripheral blood lymphocytes and Raji cells were incubated in phosphate-buffered saline for 1 hr under tissue culture conditions, gC1q-R was released only from Raji cells, suggesting that perhaps activation or transformation leading to immortalization is required for gC1q-R release. Subcellular fractionation of Raji cells and analyses by enzyme-linked immunosorbent assay and Western blotting showed that the two molecules are present in the cytosolic fractions as well as on the membrane. The data suggest

that soluble forms of both C1q-binding molecules are released from cells and that these molecules may play important roles *in vivo* as regulators of complement activation. © 1997 Academic Press

INTRODUCTION

The first component of complement, C1, is present in blood as a pentameric complex consisting of the Ca^{2+} -dependent $\text{C1r}_2\text{C1s}_2$ tetramer and the collagen-like hexameric glycoprotein C1q (1, 2). Within the complex, C1q serves as the recognition unit during activation of the classical pathway by binding to polymeric structures such as circulating immune complexes, bacteria, viruses, and other activating substances (2). Binding initiates a series of intramolecular changes that ultimately activate the proenzymes, C1r and C1s. Then, the C1 inhibitor removes activated C1r and C1s (3), liberating C1q in a form that is capable of interacting with the cell surface of various blood cells such as B cells, monocytes, neutrophils, eosinophils, platelets, and endothelial cells (4-11). Structurally, C1q consists of two unique domains: the globular "heads" (gC1q), whose major function is thought to provide binding sites for activators of the classical pathway (2), and a collagen-like "stalk" (cC1q) which is capable of interacting with diverse molecular species including collagen, laminin, fibronectin, and C-reactive protein (reviewed in 11, 12). Since C1q within multimeric C1 does not efficiently bind to cells, it is postulated that dissociation of activated C1r and C1s from C1q exposes sites in both of these domains that are necessary for the binding of C1q to cell surfaces.

To date, three types of C1q-binding cell surface molecules have been described and characterized. Two of these, a 60-kDa (4, 5) and a 100-kDa (13) glycoprotein, bind to the "collagen-like" region of C1q, and the other, a 33-kDa molecule, has affinity for the globular heads (14). Whereas the 100-kDa (SDS-PAGE, nonreducing) molecule appears to be uniquely expressed on mono-

¹ This work was supported in part by Grants 94405 from the American Heart Association-N.Y. Affiliate, 0001 from the Burroughs Wellcome Research Travel Fund, and IM771 from the American Cancer Society (B.G.) and RO1HL5029101 from the National Heart, Lung, and Blood Institute (E.I.B.P. and B.G.). The authors are grateful to Ms. Rebecca Rowehl (Molecular Genetics and Microbiology) for growing large batches of Raji cells and Tom Fischer (Center for Analysis and Synthesis of Macromolecules) for synthesizing the synthetic peptides in a timely fashion.

cytes and neutrophils (12), the 60-kDa and 33-kDa molecules which are referred to as cC1q-R² and gC1q-R, respectively, are coexpressed on a wide range of tissues and cell types (14). In addition to C1q, the 60-kDa cC1q-R which by itself is a surface-expressed, glycosylated homolog of the high-affinity calcium-binding protein calreticulin (15, 16), is postulated to serve as a receptor for molecules which are structurally similar to C1q (17). These molecules include mannan binding lectin, lung surfactant protein A, and conglutinin, are lectin-like molecules which contain collagen-like sequences, and are presumed to bind to cC1q-R through these domains (17). Similarly, the gC1q-R molecule has been shown recently to bind to proteins of the blood coagulation system including high-molecular-weight kininogen and Hageman factor (Factor XII) and may represent a naturally occurring initiator of the intrinsic blood coagulation and kinin-generating pathway on the endothelial cell surface (18).

Preliminary experiments performed in our laboratory (19) had indicated that soluble forms of cC1q-R and gC1q-R are present in serum as well as synovial fluid. In this report we show that both cell surface proteins are released from activated lymphocytes and have similar functional and biochemical properties as their membrane counterparts.

MATERIALS AND METHODS

Chemicals and reagents. The following chemicals and reagents were purchased from the sources indicated: Fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT); RPMI 1640, DMEM, Medium 199, HBSS (Hank's balanced salt solution), 100× antibiotic-antimycotic mixture (GIBCO-BRL, Gaithersburg, MD); AP-GAM-Fc- and Fab-specific, AP-STRAV (alkaline phosphatase-conjugated streptavidin), AP-XTRAV (alkaline phosphatase-conjugated ExtrAvidin), pNPP (*p*-nitrophenyl phosphate, 5-mg tablets), DMSO (dimethyl sulfoxide) (Sigma Immunochemicals, St. Louis, MO); NHS-LC-biotin (sulfo succinimidyl-6-[biotinamido] hexanoate), ImmunoPure A/G IgG purification and ImmunoPure F(ab')₂ kit, Tween 20 (Pierce Chemical Co., Rockford, IL); PD-10 columns, Ficoll-Paque (Pharmacia Biotech. Inc., Uppsala, Sweden); NBT (nitro blue tetrazolium), BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD); PEG (polyethylene glycol 1500 (Boehringer-Mannheim).

Cultured cells. Most of these studies were performed using Raji, a cell line originally derived from

Burkitt's lymphoma (20) and from which the 60- and 33-kDa C1q receptor was originally isolated and characterized (5, 14). The cells were grown in RPMI 1640 containing 10% heat-inactivated (3 hr, 56°C) FCS and 1% of an antibiotic-antimycotic stock mixture (100×) consisting of penicillin G (10,000 U/ml), amphotericin B (25 µg/ml), and streptomycin sulfate (10,000 µg/ml) and maintained in a humidified 37°C incubator in an atmosphere of 95% air and 5% CO₂.

Preparation of peripheral blood lymphocytes (PBL). Highly purified peripheral blood lymphocytes (PBL) were prepared from EDTA-anticoagulated blood of healthy donors by the method of Boyum (21). The blood was diluted 1:3 in 10 mM EDTA-containing PBS (PBS-E) and centrifuged on Ficoll-Paque, and the lymphocytes were taken from plasma-Ficoll interphase, removed, washed with PBS-E, resuspended in RPMI containing 10% FCS, and then adhered to plastic culture dishes to remove monocytes. The nonadherent cells were removed and washed with PBS, and after incubating them with sheep erythrocytes to remove T cells by rosetting, the cells were again subjected to centrifugation on Ficoll-Paque. The final preparation contained >90% B lymphocytes and viability as assessed by trypan blue exclusion was ≥95%.

Purification of membrane-associated, cytosolic, and released forms of C1q-R. Large amounts of Raji cells were first grown in RPMI 1640 containing 10% FCS in a 1-L roller bottle until the cell number had reached approximately 10⁶/ml. After the desired number had been achieved, the cells were pelleted by centrifugation (800g, 4°C, 10 min) under sterile conditions, washed 3 × 100 ml with RPMI 1640, and then resuspended in serum-free DMEM and grown for an additional 24-hr period. The cells were pelleted as above, and the culture supernatant was collected and saved. The cell pellet was washed and resuspended in 100 ml PBS. An aliquot was taken out to determine cell number and viability and the suspension kept frozen at -80°C. The cytosolic fraction was prepared by freeze-thawing (3×) in liquid nitrogen and collecting the supernatant after centrifugation at 45,000g (1 hr, 4°C). The pellet containing the membrane fragments was washed and used to prepare membrane proteins according to the protocols described in detail elsewhere (14) and the cC1q-R and gC1q-R were isolated as described below.

The serum-free culture supernatant (1 L) from above was concentrated 50-fold by an Amicon pressure filtration device fitted with a membrane of a molecular weight cut-off of 3500 and the concentrated supernatant which was shown by Western blotting to contain both the 33- and 60-kDa antigens was dialyzed against 10 mM sodium phosphate, pH 7.5, and 6 ml of this was applied to an HPLC Mono-Q column (5 × 5 cm) that had been precalibrated using ¹²⁵I-labeled cC1q-R and

² Abbreviations used: C1q-R, C1q receptor; cC1q-R, 60-kDa membrane protein which binds to the collagen-like "stalk" of C1q; gC1q-R, 33-kDa membrane protein which binds to the globular "heads" of C1q; gC1q-R, recombinant gC1q-R; sgC1q-R, soluble gC1q-R.

gC1q-R. After washing with equilibrating buffer, the flow rate was adjusted to 1 ml/min and the bound proteins were eluted with a NaCl concentration gradient using 1 M as a limit. The cC1q-R- and gC1q-R-containing fractions were identified by enzyme-linked immunosorbent assay (ELISA) using the respective polyclonal and monospecific antibodies.

Expression in *Escherichia coli* and purification of recombinant gC1q-R. The construction and expression of plasmid pGex-2T containing an insert encoding the mature form (MF, residues 74–282) of gC1q-R as well as the expression, purification, and characterization of the recombinant gC1q-R have been described in detail in a previous publication (14). In addition, a deletion mutant or truncated form (TF) lacking the first 22 amino acid residues has been constructed. The conditions used for the transformation of the plasmid-containing the correct TF insert into the *E. coli* strain BL 21, overexpression, purification to homogeneity, and characterization of the purified protein have also been described in detail elsewhere (22). The homogeneity of MF and TF was verified by SDS-PAGE under both reducing and nonreducing conditions as well as by Western blotting using monoclonal antibodies reactive with both forms of the recombinant gC1q-R.

Synthetic peptides. A synthetic peptide corresponding to the amino-terminal residues 18–32 of cC1q-R (cC1q-R-N₁₅) and containing two extra lysine residues has been synthesized (Advanced ChemTech, Louisville, KY). This peptide, **EPAVYFKEQFLDGDG KK**, is common to both cC1q-R and calreticulin. After purification by HPLC gel filtration, the peptide was conjugated to rabbit serum albumin (RSA) using the glutaraldehyde method (23) and the RSA-peptide conjugate was separated from excess glutaraldehyde by gel filtration on a PD-10 column followed by dialysis against PBS. The presence of peptide on the RSA molecule was verified by ELISA using a polyclonal antibody against cC1q-R (pAb-235) and a positive result was arbitrarily taken as an indication that the conjugation was successful.

In addition, two peptides corresponding to C1q A-chain residues 155–164 (**SSSRGQVRRS**) and a control peptide in which the two arginine residues at positions 162 and 164 were substituted by glutamine (**SSSRGQVQQS**) were synthesized.

Monoclonal and polyclonal antibodies to cC1q-R and gC1q-R. The production and purification of polyclonal antibodies against the 33-kDa "native" gC1q-R (pAb-237) or the gC1q-R peptide (pAb 274) corresponding to the NH₂-terminal peptide (residues 76–93) have been described previously (14). Recently, a panel of IgG monoclonal antibodies which react with several unique epitopes on the gC1q-R molecule have also been pro-

duced and described (24). Similarly, polyclonal antibodies reactive with cC1q-R (pAb-235) have been produced and described in an earlier publication (14). Anti-cC1q-R peptide (pAb-560) was produced by immunizing rabbits with RSA-conjugated cC1q-R-N₁₅ and following the protocol and immunization schedule described for the production of anti-gC1q-R peptides (14). The presence of anti-cC1q-R peptide antibody was assessed by ELISA and Western blotting using whole cC1q-R and RSA-conjugated or unconjugated (ELISA) cC1q-R-N₁₅. The IgG fraction from each antiserum or culture supernatant of anti-gC1q-R hybridomas was purified by ammonium sulfate precipitation followed by purification using the ImmunoPure A/G IgG purification kit (Pierce). IgG fragments were generated using the ImmunoPure F(ab')₂ preparation kit (Pierce) following the manufacturer's recommendations.

Protein determination. Protein concentrations were determined by the method of Bradford (25), and for solubilized membrane proteins which contained non-ionic detergents, the detergent-compatible, bicinchoninic acid-dependent protein assay was used (26).

Protein biotinylation. Proteins were biotinylated as described earlier (27) using NHS-LC-biotin. After removal of the excess and free biotin by gel filtration on a G-25 column, the degree of biotinylation was evaluated by ELISA using AP-XTRAV as a probe, and the proteins were aliquoted and kept frozen at –80°C.

Cell surface biotinylation of Raji cells. Surface biotinylation of cells essentially followed the procedure described by Cole *et al.* (28). Briefly, cells to be labeled were washed three times in cold PBS, pH 7.4, and then resuspended in the same buffer at a concentration of 10²/ml. Biotinylation was initiated by incubation of cells with 5 mM (final concentration) NHS-LC-biotin. After 1 hr at 4°C, the cells were washed three times with cold PBS, an aliquot was taken out for determination of viability by trypan blue exclusion, and the cells were resuspended in 1 ml of lysis buffer (20 mM Na₂HPO₄, pH 7.4) containing 1% NP-40 and a cocktail of enzyme inhibitors and left on ice for 1 hr. The nuclei and insoluble cellular debris were removed by centrifugation (1 hr, 45,000g, 4°C), the protein concentration of the supernatant containing the labeled proteins was determined, and the proteins were then aliquoted and kept frozen at –80°C.

ELISA. The ability of the soluble C1q-binding proteins to bind C1q was assessed by solid-phase ELISA using microtiter plates (MaxiSorb, Nunc, Denmark). Briefly, microtiter wells were first precoated (2 hr, 37°C or overnight, 4°C) with 250 ng/well C1q. All dilutions for coating were made in carbonate buffer, pH 9.5 (15 mM Na₂CO₃ and 35 mM NaHCO₃). After blocking the

unreacted sites by incubating (1 hr, 37°C) with 300 μ l of 1% BSA in TBS (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl), various dilutions of the biotin-labeled C1q-binding proteins were added and incubated for 1 hr at 37°C. The bound biotinylated antigens were probed by addition of 50 μ l of a predetermined dilution of AP-STRAV in TBS-B (TBS plus 0.1% BSA) followed by a 30-min incubation with 50 μ l of a 1 mg/ml pNPP substrate solution freshly dissolved in 10% diethanolamine, pH 9.5, containing 5 mM MgCl₂ and 0.02% NaN₃. Alternatively, unlabeled proteins were incubated with the C1q and the bound proteins probed with either mAb 60.11 anti-gC1q-R or pAb-560 anti-cC1q-R. The absorbance of the resulting color development was measured at 405 nm using a Dynatech MR 700 ELISA plate reader. Washes between all reactions were done 3 \times 2 min in TBS-T (TBS plus 0.05% Tween 20) and the wells completely dried before addition of the next reagent. Unless otherwise stated, all ELISA experiments were carried out in duplicate wells.

SDS-PAGE and Western blot analysis. Solubilized cell membranes or purified proteins were applied to each lane of a 1.5-mm-thick slab of a 9% SDS-PAGE (29) and the proteins separated by electrophoresis under nonreducing conditions. The electrophoretically separated proteins were then electrotransferred to polyvinylidene difluoride (PVDF) nitrocellulose membrane (30), the membrane sites blocked with 5% nonfat milk containing TBS-T (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20), and the bound proteins probed with predetermined dilutions of the appropriate antibodies or nonimmune species-matched IgG. The bound antibodies were visualized by an appropriate dilution (in TBS-B) of AP-GAM or AP-GAR followed by reaction with a mixture of NBT-BCIP in color development solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂) such that the final BCIP was 0.3 mg/ml and NBT was 0.15 mg/ml. After 30 min, color development was stopped by washing the filter membranes with color stop solution (20 mM Tris-HCl, pH 2.9, containing 1 mM EDTA).

Receptor blot analysis. To identify to which chains of C1q the cC1q-R or gC1q-R molecules bind, a ligand blot analysis was performed. To this effect, highly purified C1q (5 μ g/lane) was first run on a 9% SDS-PAGE under reducing and nonreducing conditions and electrotransferred onto PVDF membranes and the membrane-bound C1q was probed with either biotinylated gC1q-R or cC1q-R followed by development with AP-XTRAV followed by NBT-BCIP as described for the Western blot. Controls included lanes in which the C1q was probed with a comparable dilution of biotinylated BSA instead of the receptor protein.

Antigen-capture ELISA. For the antigen-capture assay, microtiter plates were first coated with a prede-

termined optimal concentration of capturing mAb 60.11 anti-gC1q-R antibody (overnight, 4°C) or anti-cC1q-R-N₁₅ peptide (pAb-560), washed with TBS-T, and blocked with 1% BSA before addition of gC1q-R-containing solution in 0.1% BSA. After an hour at 37°C, the captured antigen was probed with a known concentration of rabbit anti-gC1q-R (pAb 237) or anti-cC1q-R (pAb-235) and the ELISA procedure carried out as described above. A standard for the capture assay included concentrations (0–1000 ng/ml, in TBS-B) of highly purified gC1q-R and cC1q-R or a similarly treated irrelevant antigen, BSA.

Hemolytic assay. Standard hemolytic assays (31) were carried out as follows. For assays with gC1q-R, concentrations (0–20 μ g) of either recombinant (rgC1q-R) or soluble (sgC1q-R) gC1q-R were first incubated (1 hr, 37°C) with 10- μ l normal human serum (NHS) in a total volume of 100 μ l of GVB (0.1% gelatin containing veronal buffer, pH 7.5). The volumes of the mixtures were brought to 950 μ l with GVB and incubated for an additional hour with 50 μ l EA [sheep erythrocytes (E) 1.5×10^8 /ml, sensitized with anti-E antibody (A)]. The degree of lysis was then assessed by spectrophotometric measurement of hemoglobin in the supernatants. For assays in which the effect of cC1q-R was evaluated, concentrations of cC1q-R (0–20 μ g) were first preincubated with EDTA-serum (10 mM EDTA final concentration). After incubation, 20 mM CaCl₂ was added to the reaction mixture to reassemble the C1 complex and incubated for 15 min at 37°C and the hemolytic assay performed as above. Heat-aggregated IgG (agg.IgG) was used as a positive control and the results are given as percentages of the total releasable hemoglobin affected by lysis of EA with H₂O.

RESULTS

Purification of soluble cC1q-R and gC1q-R. Concentrated Raji cell culture supernatant (6 ml) was applied onto an HPLC fitted with a Mono-Q column (5 \times 5 cm) as described and after washing, the flow rate was adjusted to 1 ml/min and the bound proteins were eluted with a 0–2 M NaCl concentration gradient. The C1q receptor proteins were identified by ELISA using monospecific polyclonal antibodies reactive with the respective antigens. As shown in Fig. 1, two discrete peaks were identified. The first peak which eluted at 37.5 min and a NaCl concentration of 0.4 M was reactive with anti-cC1q-R, whereas the peak corresponding to gC1q-R eluted at 43.8 min and a NaCl concentration of 0.7 M. The two peaks were pooled separately, concentrated, and further purified by passage over the same Mono-Q column. Such a purification scheme yielded homogeneous preparations of cC1q-R and gC1q-R. Comparison of these antigens to their membrane coun-

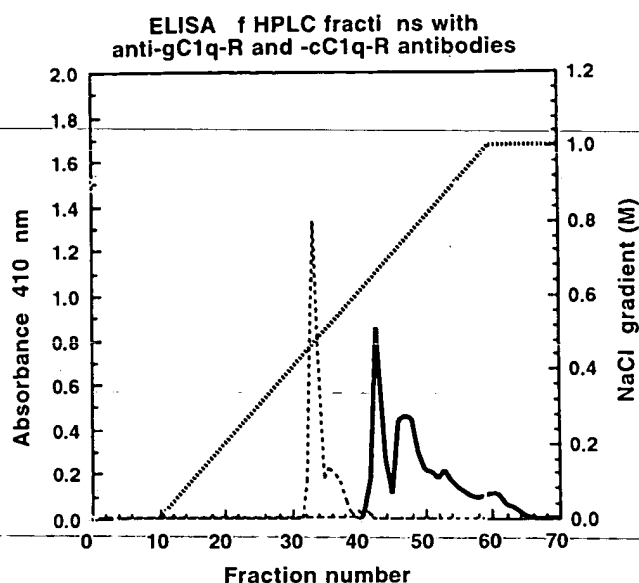


FIG. 1. HPLC profile of soluble C1q-R purification. Concentrated serum-free culture supernatant from Raji cells was dialyzed against 10 mM sodium phosphate, pH 7.5, and 6 ml subjected to HPLC purification using a Mono-Q column (5 × 5 cm) that had been equilibrated with the same buffer. After washing, the flow rate was adjusted to 1 ml/min, the bound proteins were eluted using a concentration gradient of 0–1 M, and 1-ml fractions were collected. The C1q-Rs were detected using their respective monospecific antibodies. The positions of cC1q-R-containing fractions which eluted at 37.5 min and a NaCl concentration of ~0.4 M are indicated by a dashed peak, whereas the gC1q-R peak which eluted at 43.8 min after the NaCl concentration had reached ~0.7 M is indicated by a solid line.

terparts did not reveal appreciable differences in their apparent molecular weights when analyzed by SDS-PAGE and Western blotting (Fig. 2).

The identity of the purified antigens was further confirmed by comparing their N-terminal sequences to the known sequences of membrane gC1q-R and cC1q-R. The first 10 residues of each soluble protein were found to be identical to those of their membrane counterparts (not shown).

Binding of soluble cC1q-R and gC1q-R to C1q. To verify whether the soluble proteins retained their ability to bind C1q, solid-phase binding assays were performed. Briefly, both proteins were biotinylated as described under Materials and Methods and then reacted with C1q-coated microtiter plates. As shown in Fig. 3, both biotinylated proteins were able to bind to C1q in a dose- and ionic strength-dependent manner, with the gC1q-R molecule binding with higher affinity than the cC1q-R, consistent with previous observations (14).

Both cC1q-R and gC1q-R bind to the A-chain of C1q. Using a receptor blot analysis, an experiment was performed to identify the chains of C1q to which each of the soluble receptors would bind. Although both molecules

were found to bind to denatured as well as nondenatured C1q, only the results obtained with the gC1q-R blot are shown here (Fig. 4). Under these conditions, gC1q-R binds well to the A-chain of C1q and moderately to the C-chain of C1q.

Since in earlier studies (14) we have shown that gC1q-R inhibits complement activation by binding to C1q and thus prevents the immune complexes from binding to the globular heads of C1q, we had hypothesized that the binding sites for gC1q-R and that of immune complexes on the C1q globular heads may overlap with each other or even be identical. Since the arginine residues at positions 162 and 163 of the A-chain have been implicated as the major binding sites for IgG, we generated two synthetic peptides to test this hypothesis. The test peptide corresponded to A-chain residues 155–164 (SSSRGQVRRS), whereas the control peptide was identical except that the arginine residues at positions 162 and 163 were substituted by glutamine (SSSRGQVQQS). As shown in Fig. 5, whereas biotinylated gC1q-R bound strongly to microtiter wells coated with the test peptide, no binding was observed with the control peptide and it did not make any difference whether the incubations were made in TB or TBS. Other peptides such as A-chain residues 14–26 which contain arginine residues (AGRPGRRRGRLK) did not support binding of gC1q-R (not shown). Similar results were obtained when sgC1q-R was used instead of rgC1q-R.

Inhibition of C1q hemolytic activity. In previous studies, we had shown that cC1q-R inhibits the lysis

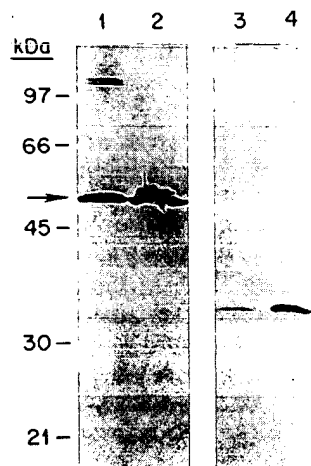


FIG. 2. SDS-PAGE analysis of soluble and membrane C1q-Rs. The two C1q-R peaks from Fig. 1 were pooled separately, concentrated, and repurified on the same Mono-Q column and the homogeneity of the proteins was analyzed by SDS-PAGE and Coomassie staining. Lanes 1 and 2 are cC1q-R (~10 µg/lane), whereas lanes 3 (~3 µg) and 4 (~5 µg) show gC1q-R. Lanes 1 and 3 are membranes cC1q-R and gC1q-R, respectively, whereas, lanes 2 and 4 represent the respective purified soluble proteins.

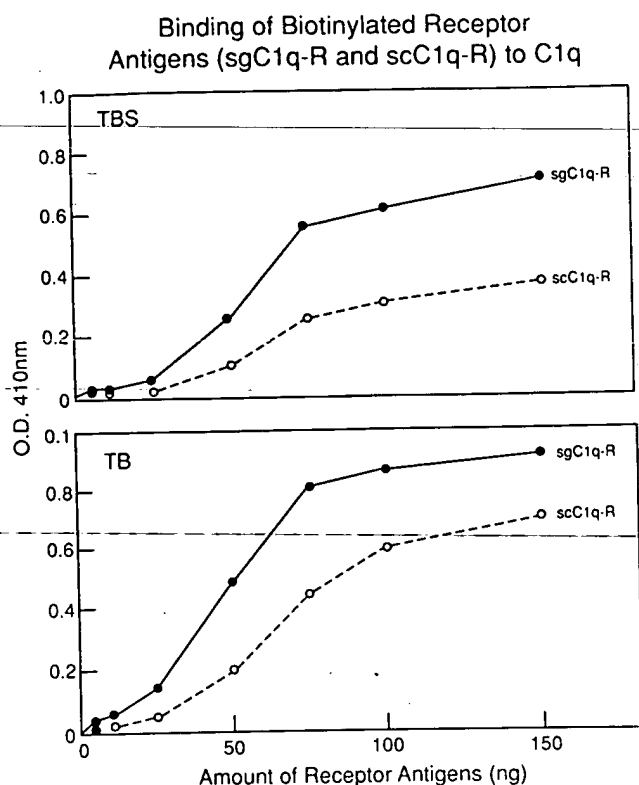


FIG. 3. Binding of soluble cC1q-R and gC1q-R to C1q. The ability of the purified soluble receptor antigens to bind to C1q was assessed by solid-phase ELISA. Wells that had been precoated (overnight at 4°C) with 250 ng/well C1q were reacted (2 hr, 37°C) with varying concentrations of either biotinylated scC1q-R or sgC1q-R in Tris buffer (TB) or Tris-buffered saline (TBS). The bound antigens were then detected by AP-STRAV as described under Materials and Methods. Each data point is a mean of two experiments run in triplicate and after the value for biotinylated BSA used as a nonspecific antigen instead of the receptor proteins had been subtracted.

of EA by normal human serum (5). More recently, we also demonstrated that gC1q-R (14) is capable of inhibiting serum C1q hemolytic activity by competing with

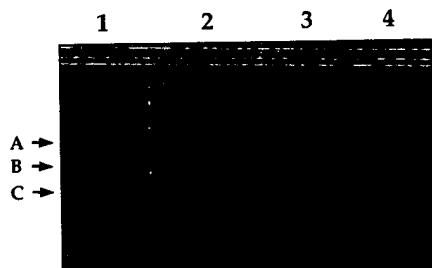


FIG. 4. Receptor blot analysis. Several lanes of C1q (5 μ g/ml) were first run on a 9% (w/w) SDS-PAGE with reduction and the individual lanes analyzed by "receptor" blot using biotinylated cC1q-R (lane 2), gC1q-R (lane 3), or BSA (lane 4). Lane 1 is C1q stained with Coomassie blue to show the individual chains of C1q.

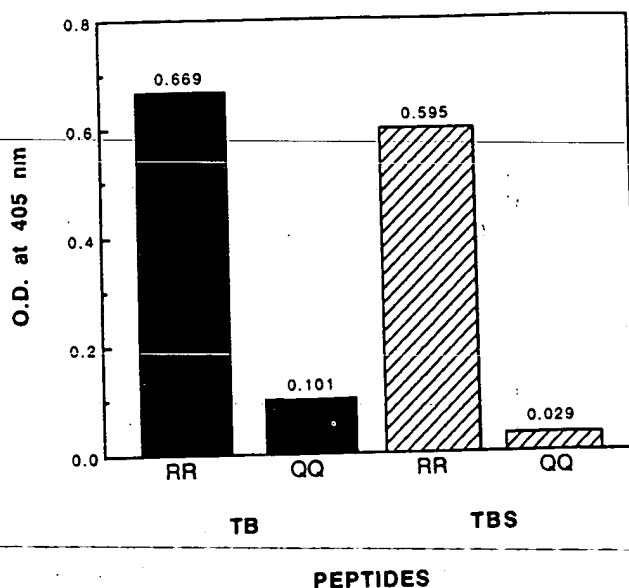


FIG. 5. Binding of biotinylated recombinant gC1q-R to C1q-A-chain peptide (RR) but not to a control peptide (QQ). ELISA wells were first coated (2 hr, 37°C) with 50 μ l (1 mg/ml, CO₂, pH 9.6) of peptides for 2 hr at 37°C followed by overnight at 4°C. After blocking, biotinylated gC1q-R (1 μ g/ml, TBS) was added and the bound receptor antigen detected with alkaline phosphatase-conjugated streptavidin. The data bars are means of three experiments run in duplicate after the values for nonspecific binding to BSA were subtracted.

immune complexes for the same binding site on C1q (32). In the present study we show (Fig. 6) that soluble gC1q-R (sgC1q-R) was also capable of inhibiting C1q hemolytic activity and its efficiency was similar to that of the recombinant form of gC1q-R (rgC1q-R).

Subcellular and extracellular analysis to identify localization of C1q receptors in Raji cells. To determine the distribution of gC1q-R and/or cC1q-R within subcellular or extracellular fractions, Raji cells were first grown to confluence in serum-free culture medium. Then, the cells were harvested and the culture supernatant was retained and concentrated 12-fold. Preparations of cytosolic and membrane proteins from the cells were compared in parallel with the culture supernatant by Western blotting after the proteins had been run on SDS-PAGE under reducing conditions. The anti-gC1q-R mAb 60.11 recognized a protein band of approximately 33 kDa in all three fractions (Fig. 7). Similarly, the pAb anti-cC1q-R specifically recognized the 60-kDa band of cC1q-R (Fig. 8) in all three fractions. However, neither the nonimmune mouse IgG nor the nonimmune rabbit IgG detected either band in the membrane fraction or in the culture medium under the same conditions. Furthermore, both receptors were found to be present in significant concentrations in the cytosolic fraction and at lower, but detectable levels in the membrane fraction and the culture supernatant of proliferating Raji cells.

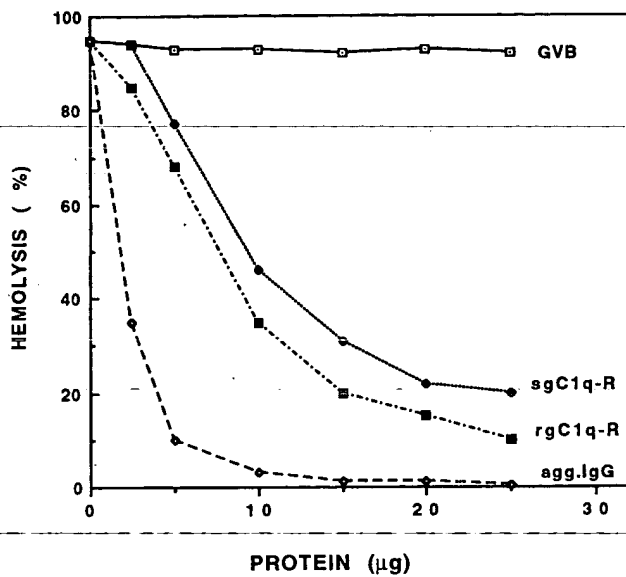


FIG. 6. Inhibition of hemolytic activity. NHS (10 μ l) was first incubated (1 hr, 37°C) with either GVB or concentrations of agg.IgG, sgC1q-R, and rgC1q-R in a total volume of 100 μ l GVB. After incubation, the volume of each tube was brought to 950 μ l with GVB and the mixture further incubated with 50 μ l of EA. Cell lysis was assessed by spectrophotometric measurement of hemoglobin in the supernatants. Each data point is a mean of three experiments run in duplicate.

Demonstration of soluble C1q receptors released from Raji cells or isolated human lymphocytes. Raji cells or freshly isolated human lymphocytes were suspended at a concentration of 10^7 cells/ml in PBS containing Ca^{2+} and Mg^{2+} and incubated under tissue culture con-

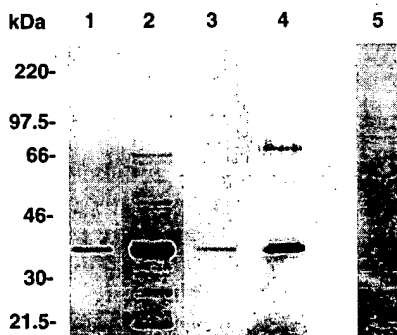


FIG. 7. Western blot analysis of distribution of gC1q-R. Raji cell cultures were pelleted and lysed and the membrane and cytosolic fractions solubilized as noted under Materials and Methods. Each subcellular fraction was loaded at a volume equivalent to 3×10^6 cells and run in parallel with an equivalent volume of the cell- and serum-free culture supernatant under reducing conditions: lane 1, membrane proteins; lane 2, cytosolic proteins; lane 3, culture supernatant; and lane 4, recombinant gC1q-R. The blot was probed with monoclonal antibodies to gC1q-R. Lane 5 was loaded with the identical volume of the cytosolic fraction and then probed with nonimmune mouse IgG as a control.

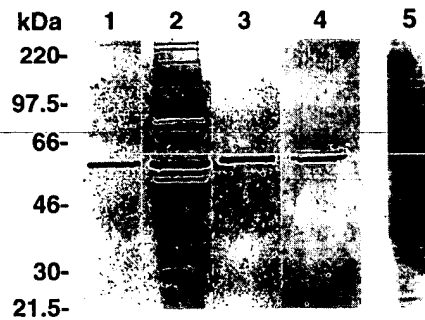


FIG. 8. Western blot analysis of distribution of cC1q-R. The experiment was run as described in the legend to Fig. 7 except that the blot was probed with anti-cC1q-R pAb-560: lane 1, membrane proteins; lane 2, cytosolic proteins; lane 3, culture supernatant; and lane 4, purified cC1q-R. Lane 5 was loaded with an identical volume of the cytosolic fraction and then probed with nonimmune rabbit IgG as a control.

ditions for 1 hr. The cells were then pelleted, the supernatant was saved, and the cell pellet was used to prepare membrane proteins. The gC1q-R and cC1q-R contents of the membrane fractions from each cell type were then compared to the presence of each receptor in the incubation buffer by Western blot analysis. Duplicate SDS-PAGE gels were run and the proteins transferred to PVDF membrane. One blot was exposed to anti-gC1q-R mAb 60.11 and the other to anti-cC1q-R pAb-560. As expected, both receptors were present in the membranes of Raji cells and peripheral blood lymphocytes at about the same concentration (not shown). However, while the cC1q-R protein was found in the cell suspension buffer from incubations of both Raji cells and the lymphocytes, the gC1q-R protein was only present in supernatants from the Raji cell culture (Fig. 9). ELISA of the same samples using the same antibodies also detected very little gC1q-R in the supernatant from the lymphocyte culture, thereby confirming the Western analysis.

Presence of cC1q-R and gC1q-R in serum. Antigen-capture assay for the presence of soluble receptor antigens demonstrated that gC1q-R was present in small but detectable amounts (50–100 ng/ml) in serum as well as other body fluids such as tears and saliva, with tears containing more gC1q-R than other samples tested (Table 1). A similar pattern (45–130 ng/ml) of distribution was observed when the samples were assessed for the presence of cC1q-R, with tears containing consistently the highest level (130 ± 35 ng/ml).

DISCUSSION

The presence of membrane proteins which bind to the two functional domains of C1q is now well documented (3, 14, 33). Recent reports have suggested

that cC1q-R and gC1q-R can be released from neutrophils when stimulated by phorbol esters (19) and that calreticulin, a previously presumed cytosolic protein and a homolog of cC1q-R, is secreted *in vitro* from several cell types (15, 35) and has been detected in human plasma (36). The present studies were therefore undertaken to examine the conditions under which these C1q-binding proteins are released and the role that they might play in regulating C1q functions. The data demonstrate that Raji cells as well as peripheral B lymphocytes contain significant amounts of gC1q-R and cC1q-R in the cytosol as well as on their membranes. In addition, reasonable levels of both receptors have been found in the culture supernatant. More importantly, however, this report documents for the first time that while resting peripheral blood lymphocytes release quantifiable amounts of cC1q-R, they do not release detectable gC1q-R. This suggests that the gC1q-R gene may be an important molecule which is targeted for upregulation by proliferation-inducing agents. Since Raji cells are Epstein virus (EBV)-transformed lymphoblastoid cells, it is hypothesized that viral elements which induce a proliferative response may also upregulate gC1q-R secretion. Consistent with this hypothesis is the finding (not shown) that supernatants of a number of C1q-R-bearing, virus-transformed cultured cells tested such as Daudi and Wil2-WT cells showed the presence of gC1q-R and cC1q-R by Western blot analysis.

Western blot analysis under reducing conditions using a polyclonal antibody raised against an N-terminal residue common to both cC1q-R and calreticulin recog-

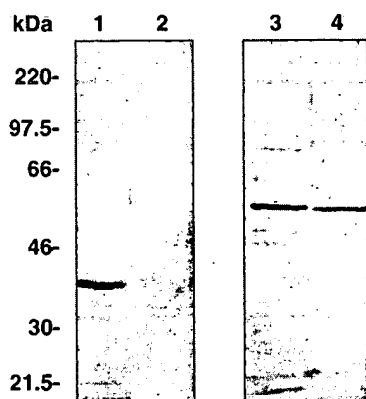


FIG. 9. Western blot analysis of C1q-Rs released from Raji cells and peripheral blood lymphocytes (PBL). Raji cells and peripheral blood lymphocytes (10^7 cells/ml) were resuspended in PBS, incubated, and pelleted and equal volumes of the supernatants were run under reducing conditions. Lanes 1 and 3 represent soluble proteins in the Raji cell supernatant, while lanes 2 and 4 are the proteins in the PBL supernatant. Lanes 1 and 2 were probed with anti-gC1q-R mAb 60.11 and lanes 3 and 4 were probed with a pAb-560 anti-cC1q-R.

TABLE 1

Quantitation of sgC1q-R Levels in Cell Medium and Body Fluids and Serum

Source	[10^6 cells] ng	[per/ml] ng
Raji	43 ± 18	—
PBL ^a	10 ± 5	—
Serum ^b	—	65 ± 24
Saliva ^c	—	35 ± 10
Tears ^d	—	129 ± 20

^a Restung, unactivated PBL.

^{b-d} Serum, saliva, and tear pools were collected from healthy volunteers, microfuged, and microfiltered using 0.22- μ m filters.

nizes a 60-kDa band in the cytosolic fraction, the membrane preparation, as well as in the culture supernatants. Since all evidence accumulated to date including functional (14, 15) and partial amino acid analysis (8, 14, 16) suggests that calreticulin and cC1q-R are highly homologous if not identical proteins, it is proposed that cC1q-R is a homolog of calreticulin. This antibody recognizes specifically a single major protein band of approximately 60 kDa in all the fractions tested (Fig. 8).

Isolation of the soluble forms of gC1q-R and cC1q-R from the culture medium yielded proteins which were immunochemically indistinguishable from their membrane counterparts (Fig. 2). Furthermore, the soluble proteins were able to bind to C1q (Fig. 3) and inhibit serum hemolytic activity either, in the case of cC1q-R, by binding to the collagen-like stalks and preventing the assembly of C1 (5) or, in the case of gC1q-R, by competing for the same binding site on the globular heads of C1q as immune complexes (Fig. 6). However, although both proteins could inhibit serum complement activity, they were unable to activate complement as assessed by C4 titration (not shown). Moreover, the findings indicate that both cC1q-R (not shown) and gC1q-R (Fig. 4) bind predominantly to the A-chain and moderately to the C-chain of C1q. In the case of gC1q-R, the binding to the A-chain appears to be mediated via two adjacent arginine residues corresponding to residues 162 and 163 as previously reported (32) and as shown in Fig. 5. That these A-chain arginine residues are involved in the binding of IgG to C1q has been previously reported (37). Chemical modification studies have also suggested the significance of arginine residues in the globular heads of C1q.³ Taken together, the results demonstrate that the membranes cC1q-R and gC1q-R and the released soluble counterparts share common physical and immunological characteristics

³ Leigh, L., Lim, B.-L., Reid, K. B. M., Dodds, A., Peerschke, E. I. B., and Ghebrehwet, B., Functional studies on gC1q-R, a novel protein which binds to the globular "heads" of C1q. IXth International Congress of Immunology, July 23–29, 1995, San Francisco.

including identical N-terminal sequences. Antibodies raised against either the N-terminal residues of gC1q-R (pAb-274) or the N-terminal residues of cC1q-R/calreticulin (pAb-560) recognize the respective membrane as well as soluble forms of these molecules. However, the mechanism by which the soluble forms are released cannot be ascertained from the present studies. It does appear, however, that agents or molecules which induce cell proliferation have the capacity to increase the amount of gC1q-R released into the surrounding milieu. Raji, a virally transformed cell line, releases both gC1q-R and cC1q-R to the culture medium. In contrast, resting peripheral blood lymphocytes when analyzed under similar conditions, release cC1q-R, but not gC1q-R. The fact that the Raji-released protein is identical to the mature form of membrane gC1q-R implies that this soluble protein is perhaps released from the membrane by site-specific enzymatic cleavage and may play an important role in cell regulation. Obviously, it will be important to determine the precise activating conditions, such as viral infection versus transformation, which result in the release of gC1q-R. Once released, the role of these receptor antigens may be either to regulate complement activation within the cell's microenvironment or to prevent the binding of C1q- or C1q-containing immune complexes to cells. In this manner proliferating cells may evade destruction or elimination by complement. Experiments are currently underway to determine whether sera from patients with proliferative disorders or fluids from patients with inflammatory conditions contain increased amounts of these receptor antigens.

REFERENCES

1. Reid, K. B. M., Chemistry and molecular genetics of C1q. *Behring Inst. Mitt.* **84**, 8-19, 1989.
2. Cooper, N. R., The classical complement pathway: Activation and regulation of the first complement component. *Adv. Immunol.* **37**, 151-216, 1985.
3. Sim, R. B., Arlaud, G. J., and Colomb, M., C1 inhibitor-dependent dissociation of human complement component C1 bound to immune complexes. *Biochem. J.* **179**, 449-457, 1979.
4. Tenner, A. J., and Cooper, N. R., Identification of cell types in human peripheral blood that bind C1q. *J. Immunol.* **126**, 1174-1179, 1981.
5. Ghebrehiwet, B., Silvestri, L., and McDevitt, C., Identification of the Raji cell membrane-derived C1q inhibitor as a receptor for human C1q. Purification and immunochemical characterization. *J. Exp. Med.* **160**, 1375-1389, 1984.
6. Malhotra, R., and Sim, R. B., Chemical and hydrodynamic characterization of the human leucocyte receptor for complement subcomponent C1q. *Biochem. J.* **262**, 625-631, 1989.
7. Peerschke, E. I. B., and Ghebrehiwet, B., Human blood platelets possess specific binding sites for C1q. *J. Immunol.* **138**, 1537-1541, 1987.
8. Peerschke, E. I. B., Malhotra, R., Ghebrehiwet, B., Reid, K. B. M., Willis, A. C., and Sim, R. B., Isolation of a human endothelial cell C1q receptor (C1q-R). *J. Leukocyte Biol.* **53**, 179-184, 1993.
9. Daha, M. R., Miltenburg, A. M. M., Hiemstra, P. S., Klar-Mohamad, N., Van Es, L. A., and Van Hinsberg, V. W. M., The complement subcomponent C1q mediates binding of immune complexes and aggregates to endothelial cells in vitro. *Eur. J. Immunol.* **18**, 783-787, 1988.
10. Kuna, P., Iyer, M., Peerschke, E. I. B., Kaplan, A. P., Reid, K. B. M., and Ghebrehiwet, B., Human C1q induces eosinophil migration. *Clin. Immunol. Immunopathol.* **81**, 48-54, 1996.
11. Ghebrehiwet, B., Functions associated with the C1q receptor. *Behring Inst. Mitt.* **84**, 204-215, 1989.
12. Tenner, A. J., Functional aspects of the C1q receptor. *Behring Inst. Mitt.* **93**, 241-253, 1993.
13. Guan, E., Burgess, W. H., Robinson, S. L., Goodman, E. B., McTigue, K. J., and Tenner, A. J., Phagocytic cell molecules that bind the collagen-like region of C1q: Involvement in C1q-mediated enhancement of phagocytes. *J. Biol. Chem.* **266**, 20345-20355, 1991.
14. Ghebrehiwet, B., Lim, B. L., Peerschke, E. I. B., Willis, A. C., and Reid, K. B. M., Isolation, cDNA cloning, and overexpression of a 33-kD cell surface glycoprotein that binds to the globular "heads" of C1q. *J. Exp. Med.* **179**, 1809-1821, 1994.
15. Eggleton, P., Lieu, T. S., Zappi, E. G., Sastry, K., Coburn, J., Zaner, K. S., Sontheimer, R. D., Capra, J. D., Ghebrehiwet, B., and Tauber, A. I., Calreticulin is released from activated neutrophils and binds to C1q and mannan-binding protein. *Clin. Immunol. Immunopathol.* **72**, 405-409, 1994.
16. Malhotra, R., Willis, A. C., Jensenius, J. C., Jackson, J., and Sim, R. B., Structure and homology of the human C1q receptor (collectin receptor). *Immunology* **78**, 341-348, 1993.
17. Malhotra, R., Thiel, S., Reid, K. B. M., and Sim, R. B., Human leukocyte C1q receptor binds other soluble proteins with collagen domains. *J. Exp. Med.* **172**, 955-959, 1990.
18. Joseph, K., Ghebrehiwet, B., Peerschke, E. I. B., Reid, K. B. M., and Kaplan, A. P., Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: Identity with the receptor which binds to the globular "heads" of C1q (gC1q-R). *Proc. Natl. Acad. Sci. USA* **93**, 8552-8557, 1996.
19. Ghebrehiwet, B., Peterson, K., Peerschke, E. I. B., Reddigari, S. R., and Reid, K. B. M., Purification and immunochemical characterization of soluble forms of the two types of C1q receptors: cC1q-R and gC1q-R. *FASEB J.* **8**, A475, 1994. [Abstract]
20. Pulvertaft, R. J. V., A study of malignant tumors in Nigeria by short-term tissue culture. *J. Clin. Pathol.* **18**, 261-273, 1965.
21. Boyum, A., Separation of lymphocytes and erythrocytes by centrifugation. *Scand. J. Lab. Invest.* **21**(Suppl.), 97, 77-79, 1968.
22. Lim, B.-L., Preissner, K., Ghebrehiwet, B., Leigh, L. E. A., and Reid, K. B. M., The binding protein for globular "heads" of complement C1q, gC1q-R: Functional expression and characterization as a novel vitronectin binding factor. *J. Biol. Chem.* **271**, 26739-26744, 1996.
23. Doolittle, K., In "Of URFs and ORFs: A Primer on How to Analyze Derived Amino Acid Sequences," p. 85, University Science Books, 1986.
24. Ghebrehiwet, B., Lu, P. D., Zhang, W., Leigh, L. A. E., Eggleton, P., Reid, K. B. M., and Peerschke, E. I. B., Epitope analysis of gC1q-R using monoclonal antibodies. *FASEB J.* **10**, A1329, 1996. [Abstract]
25. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248, 1976.
26. Smith, P. K., Krohn, R. I., Hermanson, G. I., Mallia, A. K., Gart-

- ner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C., Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85, 1985.
27. Ghebrehiwet, B., Bossone, S., Erdei, A., and Reid, K. B. M., Reversible biotinylation of C1q with a cleavable biotinyl derivative. Application in C1q receptor (C1q-R) purification. *J. Immunol. Methods* **110**, 251-260, 1988.
28. Cole, S. R., Ashmand, L. K., and Ey, P. L., Biotinylation: An alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. *Mol. Immunol.* **24**, 699-705, 1987.
29. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227**, 680-685, 1970.
30. Towbin, H., Staehlin, T., and Gordon, J., Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354, 1979.
31. Mayer, M. M., Complement and complement fixation. In "Experimental Immunochemistry" (E. A. Kabat and M. M. Mayer, Eds.), pp. 133-240, Thomas Springfield, 1967.
32. Peerschke, E. I. B., Ghebrehiwet, B., Moon, A. H., Lim, B.-L., and Reid, K. B. M., Localization of a gC1q-R recognition site in the A-chain of C1q. *FASEB J.* **9**, A807, 1995. [Abstract]
33. Bordin, S., and Page, R. C., Detection of a high-affinity binding site for the globular head regions of the C1q complement protein on a human diploid fibroblast-subtype. *Mol. Immunol.* **26**, 677-685, 1989.
34. Eggleton, P., Ghebrehiwet, B., Sastry, K. N., Coburn, J. P., Zaner, K. S., Reid, K. B. M., and Tauber, A. I., Identification of a gC1q-binding protein (gC1q-R) on the surface of human neutrophils. *J. Clin. Invest.* **95**, 1569-1578, 1995.
35. Booth, C., and Koch, G. L. E., Perturbation of cellular calcium induces secretion of luminal endoplasmic reticulum proteins. *Cell* **59**, 729-737, 1989.
36. Sueyoshi, T., McMullen, B. A., Marnell, L. L., Du Clos, T. W., and Kisiel, W. A., A new procedure for the separation of protein Z, prothrombin fragment 1.2 and calreticulin from human plasma. *Thromb. Res.* **63**, 569-575, 1991.
37. Marques, G., Anton, L. C., Barrio, E., Sanchez, A., Ruiz, S., Gaviñanes, F., and Vivanco, F., Arginine residues of the globular regions of human C1q involved in the interaction with immunoglobulin G. *J. Biol. Chem.* **268**, 10393-10402, 1993.

Received August 19, 1996; accepted with revision April 7, 1997

STIC-ILL

Adams
20

From: Romeo, David
Sent: Monday, June 04, 2001 12:36 PM
To: STIC-ILL
Subject: 09503421 Thielens N M; Bersch B; Hernandez J F; Arlaud G J

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Thielens N M; Bersch B; Hernandez J F; Arlaud G J
TI ~~Structure and functions of the interaction domains of C1r and C1s:~~
keystones of the architecture of the C1 complex.
SO IMMUNOPHARMACOLOGY, (1999 May) 42 (1-3) 3-13. Ref: 40
Journal code: GY3; 7902474. ISSN: 0162-3109.
AN 1999334845

ADONIS - Electronic Journal Services

Requested by

Adonis

Article title	Structure and functions of the interaction domains of C1r and C1s: keystones of the architecture of the C1 complex
Article identifier	0162310999104892
Authors	Thielens_N_M Bersch_B Hernandez_J-F Arlaud_G_J
Journal title	Immunopharmacology
ISSN	0162-3109
Publisher	Elsevier Netherlands
Year of publication	1999
Volume	42
Issue	1-3
Supplement	0
Page range	3-13
Number of pages	11
User name	Adonis
Cost centre	
PCC	\$20.00
Date and time	Tuesday, June 05, 2001 3:26:28 AM

Copyright © 1991-1999 ADONIS and/or licensors.

The use of this system and its contents is restricted to the terms and conditions laid down in the Journal Delivery and User Agreement. Whilst the information contained on each CD-ROM has been obtained from sources believed to be reliable, no liability shall attach to ADONIS or the publisher in respect of any of its contents or in respect of any use of the system.



Review

Structure and functions of the interaction domains of C1r and C1s: keystones of the architecture of the C1 complex

Nicole M. Thielens^a, Beate Bersch^b, Jean-François Hernandez^a, Gérard J. Arlaud^{a,*}

^a *Laboratoire d'Enzymologie Moléculaire, Institut de Biologie Structurale Jean-Pierre Ebel (CEA-CNRS), 41, Avenue des Martyrs, 38027 Grenoble Cedex 1, France*

^b *Laboratoire de Résonance Magnétique Nucléaire, Institut de Biologie Structurale Jean-Pierre Ebel (CEA-CNRS), 41, Avenue des Martyrs, 38027 Grenoble Cedex 1, France*

Accepted 22 December 1998

Abstract

C1r and C1s, the proteases responsible for activation and proteolytic activity of the C1 complex of complement, share similar overall structural organizations featuring five nonenzymic protein modules (two CUB modules surrounding a single EGF module, and a pair of CCP modules) followed by a serine protease domain. Besides highly specific proteolytic activities, both proteases exhibit interaction properties associated with their N-terminal regions. These properties include the ability to bind Ca^{2+} ions with high affinity, to associate with each other within a Ca^{2+} -dependent C1s–C1r–C1r–C1s tetramer, and to interact with C1q upon C1 assembly. Precise functional mapping of these regions has been achieved recently, allowing identification of the domains responsible for these interactions, and providing a comprehensive picture of their structure and function. The objective of this article is to provide a detailed and up-to-date overview of the information available on these domains, which are keystones of the assembly of C1, and appear to play an essential role at the interface between the recognition function of C1 and its proteolytic activity. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Complement; C1; Serine proteases; Protein modules; Protein–protein interaction; Calcium binding

1. Introduction

The protease that triggers the classical pathway of human complement, C1, is a multimolecular com-

plex resulting from the noncovalent association of two distinct entities: the recognition protein C1q, and the catalytic subunit C1s–C1r–C1r–C1s, a Ca^{2+} -dependent tetramer comprising two different, but homologous serine proteases C1r and C1s. Through its peripheral globular domains, C1q mediates binding of C1 to target microorganisms and thereby generates a signal that triggers autolytic activation of C1r into C1r, which in turn converts C1s into C1s, the protease responsible for the highly specific proteolytic activity of C1 (see reviews by Cooper, 1985; Schumaker et al., 1987; Arlaud et al., 1998). An essential feature of C1r and C1s is that they exert their catalytic activities within the tetrameric com-

Abbreviations: CCP, protein module mostly found in complement control proteins; CUB, protein module initially found in Complement subcomponents C1r/C1s, Uegf, and Bone morphogenetic protein-1; EGF, epidermal growth factor; The nomenclature of complement proteins is that recommended by the World Health Organization; Activated components are indicated by an overbar, e.g., C1r; The nomenclature used for protein modules is that defined by Bork and Bairoch (1995)

* Corresponding author. Tel.: +33-476884981; fax: +33-476885494; e-mail: arlaud@ibs.fr

plex C1s–C1r–C1r–C1s, that itself is associated with C1q. In addition to classical serine protease domains, both proteases therefore exhibit extra, nonenzymic protein modules that endow them with specific interaction properties, including the ability to bind Ca^{2+} ions with high affinity, to associate with each other in a Ca^{2+} -dependent fashion, and to interact with C1q upon C1 assembly. C1r and C1s share homologous overall structures comprising, from the N-terminus: two CUB modules (Bork and Beckmann, 1993) surrounding a single EGF-like module (Campbell and Bork, 1993), a tandem repeat of CCP modules (Reid et al., 1986), followed by a C-terminal chymotrypsin-like serine protease domain. Although it was recognized early that the Ca^{2+} -binding and protein–protein interaction properties of C1r and C1s are mediated by their N-terminal regions (Villiers et al., 1985), precise information about the domains responsible for these properties has only been collected in the past few years.

The objective of the present article is to give a thorough and up-to-date review of the data currently available on the interaction domains of C1r and C1s, and to provide a comprehensive picture of the structure and function of these domains which appear as key elements of the assembly and activation mechanism of the C1 complex.

2. Materials and methods

2.1. Proteins

Proenzyme C1r used in the original experiments reported in this article was purified from human plasma as described by Arlaud et al. (1980). The methods used for production of the other proteins and fragments reviewed in this article are described in the corresponding original references.

2.2. Peptide synthesis and anti-peptide antibodies

Peptide C1r (129–151), overlapping the loop between residues Cys₁₂₉ and Cys₁₄₄ of the C1r EGF module, was synthesized chemically on an Applied Biosystems 430A synthesizer, using a phenylacetamidomethyl-resin and the *t*-BOC chemistry. Valine was substituted for cysteine at position 144 in

order to allow formation of a single disulfide bridge between Cys₁₂₉ and Cys₁₄₈ as this occurs in C1r, and the polymorphic site (Ser/Leu) at position 135 was occupied by a serine residue. Protecting groups for amino acid side chains were as follows: Arg (mesitylene sulfonyl), Asp, Glu (*O*-benzyl), Cys (4-methoxybenzyl), His (benzyl oxycarbonyl), Lys (2-chlorobenzyl oxycarbonyl), Ser (benzyl), Tyr (bromobenzyl oxycarbonyl). Couplings were performed by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole method, using *N*-methyl pyrrolidone and dimethylsulfoxide as coupling solvents, according to the protocol defined by Applied Biosystems. Deprotection and cleavage of the peptide from the resin was performed with trifluoromethane-sulfonic acid. Formation of the Cys₁₂₉–Cys₁₄₈ disulfide bridge was achieved by air oxidation of the peptide (0.12 mg/ml) at pH 8.2 for 20 h. The oxidized peptide was purified by reverse-phase HPLC using a linear gradient of acetonitrile (5–30%) in 0.1% trifluoroacetic acid as described by Hernandez et al. (1997). Mass spectrometry analysis of the peptide was performed as described previously (Thielens et al., 1990b), yielding a value of 2600.3 ± 0.5 Da (expected value = 2600.78).

Peptide C1r (129–151) was coupled to ovalbumin according to Tamura and Bauer (1982) and the conjugate was used to raise antibodies in rabbits. The total IgG fraction was purified by Na_2SO_4 precipitation (Prah and Porter, 1968) and specific IgGs were isolated by affinity chromatography on a Sepharose-C1r column. Production of Fab₂ and Fab fragments by pepsin and papain cleavage, respectively, was performed according to published methods (Harlow and Lane, 1988).

3. Results and discussion

3.1. Functional mapping of the interaction regions of C1r and C1s

It was shown early that, in addition to the highly restricted enzymic activities mediated by their C-terminal parts, both C1 proteases C1r and C1s also exhibit specific protein–protein interaction properties mediated by their N-terminal regions and involved, in particular, in the assembly of C1s–C1r–C1r–C1s,

the Ca^{2+} -dependent catalytic subunit of the C1 complex (Villiers et al., 1985). A number of modular fragments derived from the N-terminal part of C1r and C1s have been produced, initially by limited proteolysis, and more recently by chemical synthesis or recombinant expression methods (Fig. 1). The physico-chemical, structural, and functional data obtained on these fragments have led to a better understanding of the structure–function relationships of these regions, and of the molecular mechanisms involved in their interaction properties.

standing of the structure–function relationships of these regions, and of the molecular mechanisms involved in their interaction properties.

3.1.1. The interaction region of C1r

Initial evidence that the interaction properties of C1r are mediated by its N-terminal region came from the observation that autolytic cleavage of activated

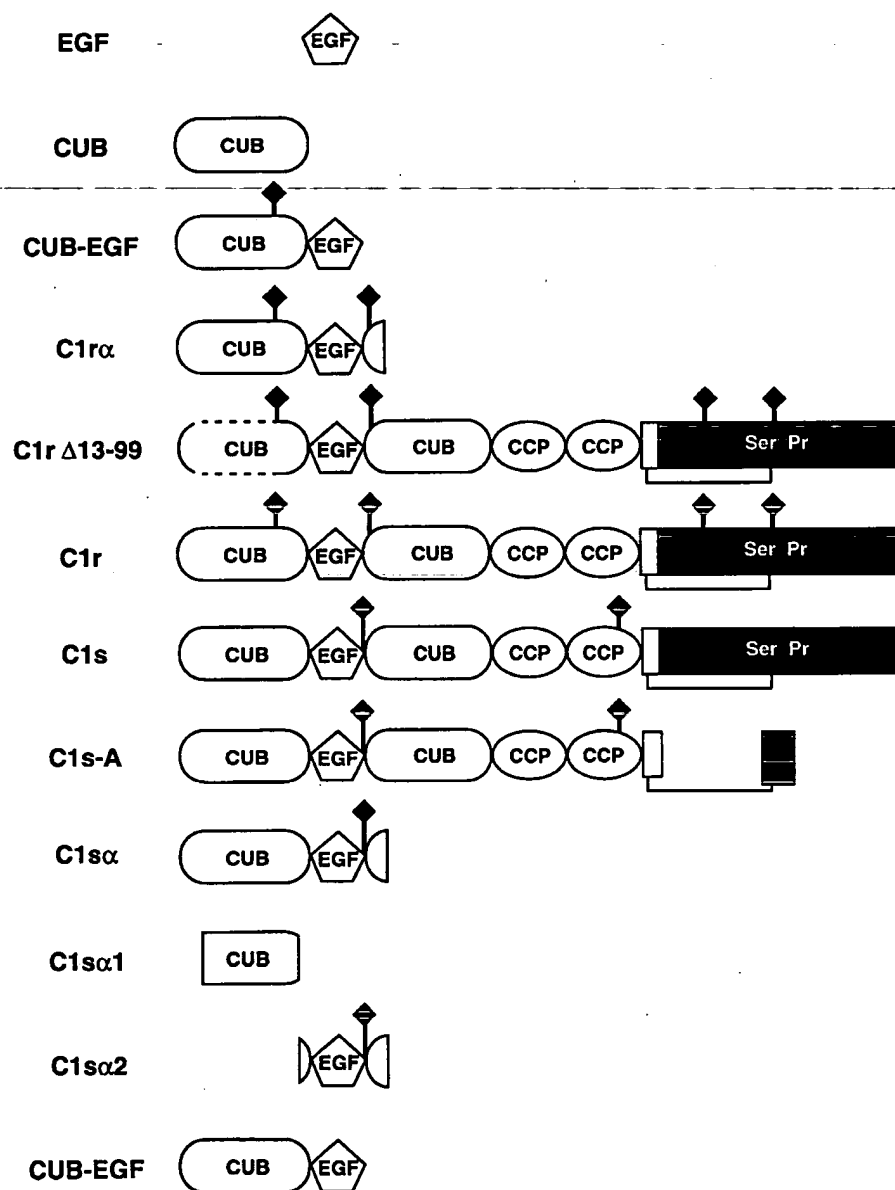


Fig. 1. Schematic representation of the modular structures of C1r and C1s, and of the various fragments derived from their N-terminal interaction regions. The nomenclature and symbols used for protein modules are those defined by Bork and Bairoch (1995). (♦) N-linked oligosaccharides. C1r Δ 13–99, deletion fragment lacking residues 13–99 of the N-terminal CUB module (Cseh et al., 1996). The N-terminal CUB fragment of C1r expressed in *Pichia pastoris* lacks glycosylation (Thielens et al., 1998).

C1r results in the removal of a large N-terminal fragment C1r α corresponding to the N-terminal half of the A-chain (Fig. 1), yielding a truncated molecule which retains the enzymic properties of intact C1r, but lacks the ability to bind Ca^{2+} ions and to associate with C1s in the presence of Ca^{2+} (Arlaud et al., 1980; Villiers et al., 1980; Villiers et al., 1985). A similar fragment C1r α was obtained by limited proteolysis of native C1r by trypsin under controlled conditions in the presence of Ca^{2+} (Busby and Ingham, 1987; Thielens et al., 1990a). This fragment was shown to extend from residues 1 to 208 of C1r, and therefore comprised the N-terminal CUB and EGF-like modules, as well as a segment corresponding to the N-terminal disulfide-loop of the following second CUB module (Fig. 1) (Thielens et al., 1990a). Fragment C1r α was found to display a low-temperature transition also observed in the whole protein, with a midpoint of 26–40°C in the absence of Ca^{2+} , shifted upward by more than 20°C upon addition of Ca^{2+} ions (Busby and Ingham, 1987). Functional characterization of C1r α indicated that it contained one high-affinity Ca^{2+} binding site ($K_D = 32 \mu\text{M}$), and retained the ability to bind C1s, as shown by the formation of Ca^{2+} -dependent C1r α -C1s and C1r α -C1s α heterodimers (Thielens et al., 1990a).

Further studies provided support to the hypothesis that C1r Ca^{2+} binding and Ca^{2+} -dependent protein-protein interaction involved structural determinants contributed by both the N-terminal CUB module and the EGF module. A deletion mutant C1r molecule lacking most of the N-terminal CUB module (residues 13–99) (Fig. 1) was expressed in a baculovirus/insect cells system and found to lack the ability to bind C1s in the presence of Ca^{2+} (Zavodszky et al., 1993; Cseh et al., 1996). The EGF module of C1r (residues 123–175) was synthesized chemically using the *t*-Boc chemistry and folded under redox conditions (Hernandez et al., 1997). The solution structure of this module was determined by two-dimensional ^1H NMR spectroscopy (Bersch et al., 1998), indicating that the C-terminal part exhibits a major and a minor antiparallel double-stranded β -sheets, i.e., a fold typical of EGF modules. In contrast, the N-terminal end of the module, as well as the unusually large (14-residue) loop between the first two cysteines (Cys₁₂₉ and Cys₁₄₄) are disor-

dered (Fig. 2). NMR spectroscopy also provided evidence that the isolated EGF module has the ability to bind Ca^{2+} , but with a K_D of about 10 mM, i.e., a value about 300 times higher than that determined for C1r α (Hernandez et al., 1997). Analysis of the chemical shift variations induced by Ca^{2+} , and modelling studies were both consistent with Ca^{2+} binding occurring through ligands (Fig. 2) homologous to those identified in the EGF modules from blood-coagulation factors IX and X, and from human fibrillin-1 (Rao et al., 1995; Downing et al., 1996; Sunnerhagen et al., 1996). The latter modules, like the C1r EGF module, all belong to a particular subset of EGF modules known to participate in Ca^{2+} binding and featuring a particular consensus sequence Asp/Asn, Asp/Asn, Gln/Glu, Asp*/Asn*, Tyr/Phe (where * indicates a β -hydroxylated residue) (Campbell and Bork, 1993). However, the decreased Ca^{2+} binding ability of the isolated C1r EGF module strongly suggested that residues located outside this module also contributed to the Ca^{2+} binding site.

To further investigate this question, the CUB-EGF module pair (residues 1–175) was recently produced using a baculovirus/insect cells expression system, and its interaction properties were studied by surface plasmon resonance spectroscopy and compared to those of other C1r fragments (Thielens et al., 1998). Using C1s as the immobilized ligand, it was shown that neither the isolated CUB and EGF fragments, nor a CUB + EGF mixture had the ability to bind in the presence of Ca^{2+} . In contrast, the CUB-EGF pair bound C1s under these conditions, with a K_D (1.5–1.8 μM) similar to that of fragment C1r α , but significantly higher than the values of 10–32 nM determined for intact C1r (Rivas et al., 1994; Thielens et al., 1998). No protein-protein interaction occurred in the absence of Ca^{2+} , and half-maximal binding to C1s was obtained at comparable Ca^{2+} concentrations for C1r (5 μM) and its CUB-EGF and C1r α fragments (10–16 μM). Using CUB-EGF as the immobilized ligand and C1s as the soluble analyte markedly increased the affinity ($K_D = 15$ –20 nM), likely due to an increased stability of the CUB-EGF fragment resulting from its covalent attachment to the surface of the sensor chip. It may be concluded from these experiments that the C1r CUB-EGF module pair is the minimal segment re-

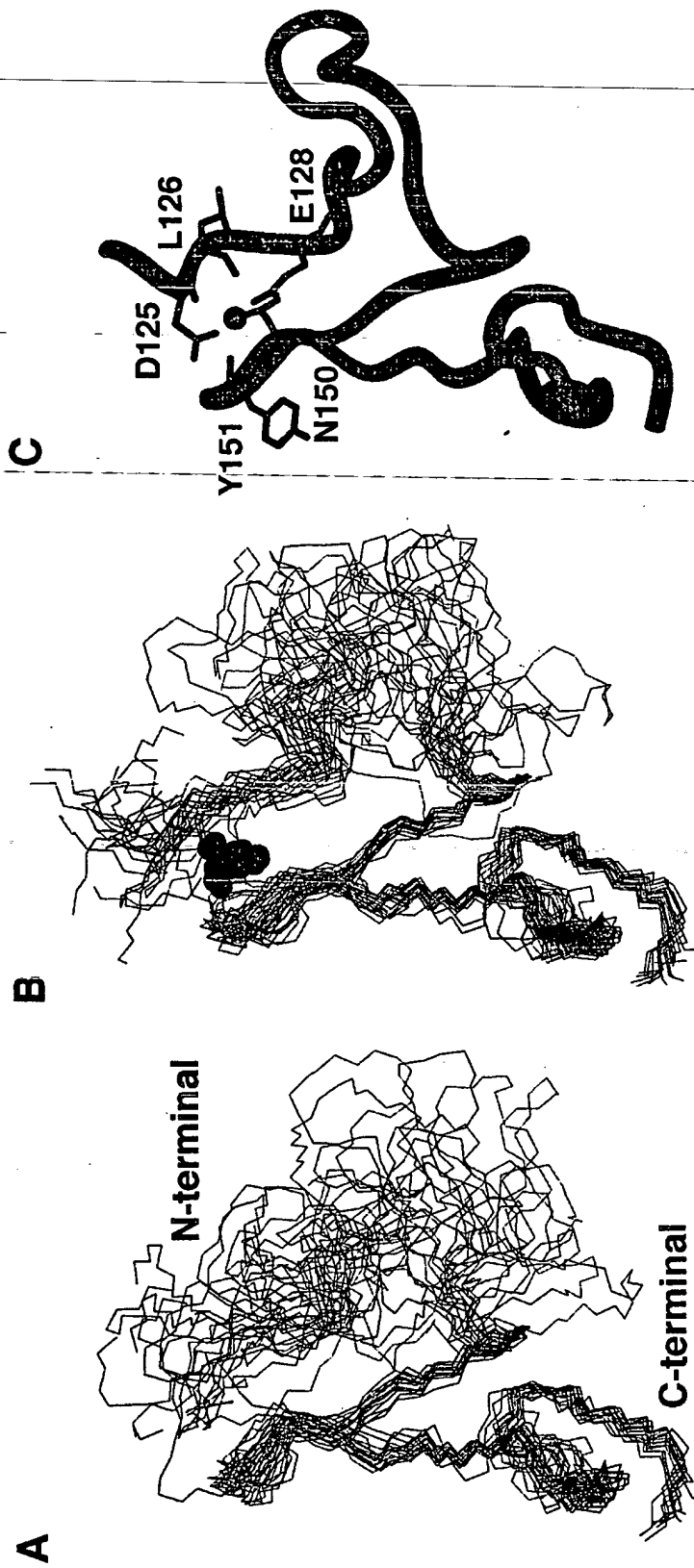


Fig. 2. Structural models of the apo- and Ca^{2+} -bound forms of the EGF module from human C1r. (A) Backbone representation of the apo-form, illustrating the mobility of the large loop between the first two cysteine residues. (B) Backbone representation of the structures of the Ca^{2+} -bound form. (C) Ribbon representation of the mean structure, featuring the Ca^{2+} -binding residues. Ca^{2+} is represented by a sphere (modified from Bersch et al., 1998).

quired for both high-affinity Ca^{2+} binding and Ca^{2+} -dependent interaction with C1s, i.e., that both functions do not involve accessory ligands located outside this segment. Other studies based on gel filtration experiments and measurements of intrinsic Tyr fluorescence provided evidence that Ca^{2+} binding induces a more compact conformation of the CUB–EGF module pair (Thielens et al., 1998). A plausible model consistent with all available data is that Ca^{2+} binding occurs primarily through ligands in the EGF module of C1r (see Fig. 2), and allows the CUB and EGF modules to move towards each other, thereby inducing formation of a compact CUB–EGF assembly. The latter likely provides the appropriate conformation as well as all of the ligands required for interaction with C1s within the C1s–C1r–C1r–C1s tetramer (Fig. 3).

3.1.2. The interaction region of C1s

It was initially shown that limited proteolysis of C1s with plasmin successively removes two N-terminal fragments $\alpha 1$ and $\alpha 2$ (Fig. 1), ending in a large

C-terminal fragment that retains esterolytic activity towards synthetic substrates, but nevertheless lacks the ability to bind C1r in the presence of Ca^{2+} , or to form Ca^{2+} -dependent homodimers as does isolated intact C1s (Villiers et al., 1985). That the C1s determinants responsible for Ca^{2+} binding and Ca^{2+} -dependent interaction are located in its N-terminal region was confirmed by isolation of an N-terminal tryptic fragment (C1s-A) lacking most of the serine protease domain and mimicking C1s ability to bind C1r in the presence of Ca^{2+} ions (Busby and Ingham, 1988). Fragment C1s-A was found to exhibit a low-temperature transition near 31°C , that was shifted to 58°C in the presence of Ca^{2+} , a feature strikingly reminiscent of that observed previously in the case of C1r (Busby and Ingham, 1987).

Shorter fragments C1s α derived from the N-terminal half of the C1s-A chain (Fig. 1) were later obtained by limited proteolysis with either trypsin or plasmin under controlled conditions (Busby and Ingham, 1990; Thielens et al., 1990a). Like its C1r counterpart, the tryptic C1s α fragment (residues 1–

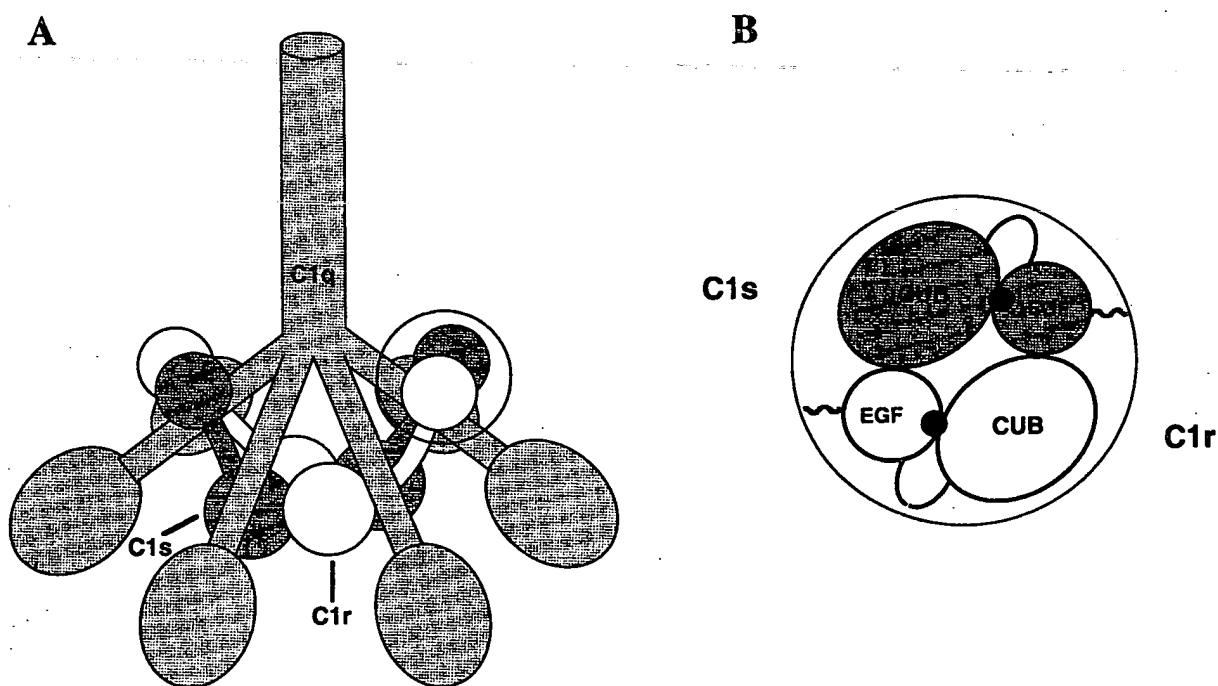


Fig. 3. The CUB–EGF module pairs of C1r and C1s in the architecture of the C1 complex. (A) Model of the C1 complex featuring interaction between the interaction domains of C1r and C1s (circled) and the collagenous stalks of C1q (modified from Arlaud et al., 1987). (B) Proposed model of the Ca^{2+} -dependent assembly of the CUB–EGF module pairs of C1r and C1s involved in the interactions between C1r and C1s and between the C1s–C1r–C1r–C1s tetramer and C1q. Calcium ions are represented by closed circles.

192) was shown to encompass the N-terminal CUB and EGF modules, plus a small segment corresponding to the N-terminal disulfide-loop of the second CUB module (Fig. 1). C1s α was found to form Ca²⁺-dependent C1s α –C1r α heterodimers, with concomitant binding of two Ca²⁺ atoms/mol, i.e., one Ca²⁺ atom per α fragment. In addition, as also observed for intact C1s, C1s α formed C1s α –C1s α homodimers that, in contrast with the C1s α –C1r α heterodimers, were found to bind three Ca²⁺ atoms/mol, i.e., one per fragment plus one extra atom at the interface. A single K_D value (about 38 μ M) for Ca²⁺ binding by C1s was determined by equilibrium dialysis (Thielens et al., 1990a), whereas analysis by sedimentation equilibrium allowed Rivas et al. (1992) to distinguish two sites with a K_D of 30 μ M and one site with a K_D of 10 nM.

Further studies provided evidence that both the CUB and EGF modules participate in Ca²⁺ binding and Ca²⁺-dependent interactions. It was shown that none of the isolated plasmin cleavage fragments α 1 (starting at Ser₂₄ and comprising most of the N-terminal CUB module) and α 2 (residues 97–195, encompassing the EGF module and a short segment from the second CUB module) (Fig. 1) retained the ability to bind Ca²⁺ with high affinity or to associate with C1r (Thielens et al., 1990b). In contrast, both α 1 and α 2 competed significantly with intact C1s for the formation of the Ca²⁺-dependent tetramer C1s–C1r–C1r–C1s. Further evidence for the implication of the N-terminal CUB module was provided by differential enzymic iodination of C1s, which indicated that residues Tyr₃₃ and Tyr₃₈ of this module were accessible in monomeric C1s, but not in the Ca²⁺-dependent C1s–C1s dimer (Illy et al., 1991). Interestingly, enzymic iodination of C1s was found to abolish its ability to form Ca²⁺-dependent homodimers, but not to impair C1s–C1r heteroassociation, confirming that these two types of interaction, also known to differ with respect to the number of Ca²⁺ atoms bound (see above) do not involve strictly identical sites and/or mechanisms. Production of a recombinant CUB–EGF module pair (C1s residues 1–159) in a baculovirus/insect cells system has recently allowed functional characterization of this segment, showing its ability to bind C1r or to dimerize in the presence of Ca²⁺ ions (Tsai et al., 1997). Although the affinities of the C1s CUB–EGF pair

for Ca²⁺ and for C1r both remain to be determined and compared to those of intact C1s, it appears very likely therefore that this segment, like its C1r counterpart, contains all of the ligands required for assembly of the Ca²⁺-dependent C1s–C1r–C1r–C1s tetramer. Whether Ca²⁺ induces in the C1s CUB–EGF pair a conformational change similar to that identified in C1r remains to be determined. However, the homology between the two module pairs and particularly the fact that the EGF module of C1s also belongs to the Ca²⁺ binding subset of EGF modules (Campbell and Bork, 1993) strongly suggests that C1s binds Ca²⁺ through a mechanism similar to that occurring in C1r, i.e., involving major ligands in the EGF-module as well as a contribution of the preceding CUB module (Fig. 3).

3.2. The C1r and C1s interaction regions in C1 architecture

Compared to the numerous studies dealing with the assembly of the C1s–C1r–C1r–C1s tetramer, less work has been carried out on the regions of C1r and C1s involved in the assembly of the C1 complex. Available information indicates that the tetramer/C1q interaction is a complex process involving multiple sites that are most likely contributed by the interaction regions of both C1r and C1s. That the N-terminal α fragment of C1s is able to ‘promote’ binding of C1r to C1q was established by Busby and Ingham (1990), who showed that a truncated tetramer C1s α –C1r–C1r–C1s α readily binds to C1q to form a pseudo C1 complex with a stability similar to that of whole C1. Similar results were obtained by Thielens et al. (1994), who showed in addition that this pseudo C1 complex is fully functional in terms of spontaneous C1r activation, indicating that the missing part of C1s, i.e., the C-terminal catalytic region, plays no role in the mechanism that triggers C1r activation in C1. It was shown more recently that the C1s CUB–EGF pair itself associates with C1r and C1q to form a complex in which C1r retains its autoactivation ability (Tsai et al., 1997). It may be concluded therefore that the CUB–EGF module pair of C1s not only contains the ligands necessary for Ca²⁺-dependent assembly of C1s–C1r–C1r–C1s, but also structural determinants required for efficient

interaction of the tetramer with C1q. This does not imply, however, that C1 assembly involves a direct contact between C1q and the CUB=EGF moiety of C1s, and there is no experimental evidence for this (Busby and Ingham, 1990; Tsai et al., 1997).

Indeed, various studies provide support to the hypothesis that C1r itself is directly involved in C1 assembly. Initial evidence for a C1q–C1r interaction came from ultracentrifugation studies showing that, unlike activated C1r, proenzyme C1r forms a complex with C1q (Lakatos, 1987). It was also shown that C1q significantly increases the activation rate of C1r in the presence of Ca^{2+} ions (Thielens et al., 1994), suggesting the occurrence of a low-affinity interaction between these proteins, and that this activating effect of C1q is abolished in the presence of 1,3-diaminopropane, a reagent known to disrupt C1 into C1q and C1s–C1r–C1r–C1s and therefore expected to act at the interface between the two subunits (Villiers et al., 1984). Other studies (Illy et al., 1991) indicated that treatment of C1s–C1r–C1r–C1s with a water-soluble carbodiimide prevents C1 assembly, through modification of acidic amino acids that are located in C1r. Taken together, the above data are consistent with the hypothesis that C1r contains a diamine- and carbodiimide-sensitive interaction site that plays a major role in C1 assembly. A plausible hypothesis is that this site lies in the CUB–EGF module pair of C1r, and that conformational changes induced in this domain upon interaction with the corresponding CUB–EGF domain of C1s enhance its affinity for C1q, thereby allowing stable interaction between C1s–C1r–C1r–C1s and C1q. It cannot be excluded, however, that the C1s CUB–EGF moiety itself contributes an accessory binding site for C1q. Also, the hypothesis that the C-terminal catalytic region of C1r may participate somehow in the interaction should be taken into consideration, given that the affinity of C1s–C1r–C1r–C1s for C1q, which remains unchanged upon activation of C1s alone, decreases significantly when C1r is activated (Villiers et al., 1982; Siegel and Schumaker, 1983; Lakatos, 1987). However, it appears likely from current data that the major interaction between the C1s–C1r–C1r–C1s tetramer and C1q is mediated by the CUB–EGF module pairs of C1r and C1s, which represent key elements of the molecular architecture of the C1 complex (Fig. 3).

3.3. Functional role of the C1r interaction region

Several studies provide strong support to the hypothesis that, in addition to its role in the assembly of C1, the interaction region of C1r is involved in the regulation of the autoactivation potential of C1r, and thereby plays a major role in C1 function. It was found earlier that Ca^{2+} ions markedly reduce, without completely inhibiting, the ability of purified C1r to autoactivate in solution (Ziccardi and Cooper, 1976; Arlaud et al., 1980). In contrast, removal by thermolytic cleavage of the N-terminal α fragment from proenzyme C1r was shown to yield a truncated molecule whose activation was totally insensitive to Ca^{2+} ions (Lacroix et al., 1989). In the same way, deletions within the interaction region of C1r were found to yield recombinant mutant proteins with a significantly increased tendency to activate (Cseh et al., 1996), providing further support to the hypothesis of a Ca^{2+} -dependent regulatory mechanism associated with the interaction region.

The occurrence of an unusually large (14 residues) loop between the first two cysteines (Cys₁₂₉ and Cys₁₄₄) of the EGF module of C1r, its high contents in charged residues, as well as its high degree of mobility as determined by NMR spectroscopy (see Fig. 2) make it a good candidate for an interaction site within C1r. These considerations prompted us to investigate the possible implication of this loop in the above-described Ca^{2+} -dependent regulatory mechanism of C1r activation. For this purpose, a peptide overlapping this loop (C1r residues 129–151) was synthesized chemically and used to generate specific anti-peptide antibodies. Fab'₂ fragments from these antibodies were then tested for their effect on the Ca^{2+} -dependent inhibition of the activation of purified C1r. As shown in Fig. 4, C1r activation proceeded rapidly upon incubation at 37°C in the absence of Ca^{2+} , and preincubation of the protein with the anti-peptide Fab'₂ fragments had no significant effect on the activation kinetics under these conditions. The activation process was slowed down in the presence of Ca^{2+} ions, whereas the Fab'₂ fragments induced further stabilization of C1r in the proenzyme state, with less than 20% activation occurring after incubation for 1 h at 37°C. Further experiments showed that monovalent Fab fragments also increased Ca^{2+} -dependent inhibition of C1r acti-

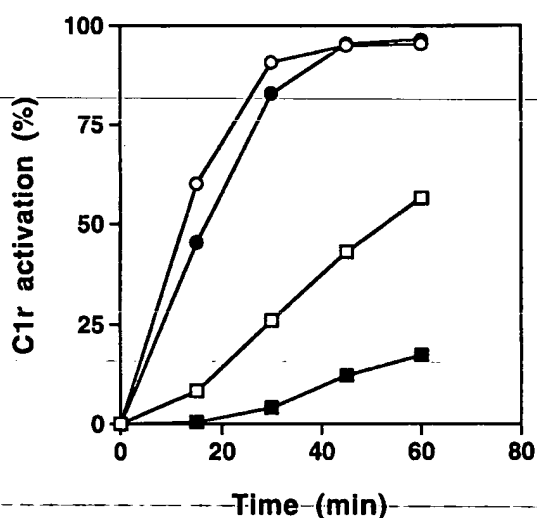


Fig. 4. Potentiation of the Ca^{2+} -dependent inhibition of C1r autoactivation by Fab_2 fragments specific of peptide C1r (129–151). Proenzyme C1r (0.13 mg/ml), either alone or after prior incubation for 4 h at 0°C with an equimolar amount of specific Fab_2 fragments, was incubated for various periods at 37°C in 145 mM NaCl, 50 mM triethanolamine-HCl, pH 7.4, in the presence of either EDTA or CaCl_2 (1 mM each). C1r activation was quantitated by SDS-polyacrylamide gel electrophoresis under reducing conditions, as described previously (Arlaud et al., 1980). (○) C1r alone in EDTA; (●) C1r+ Fab_2 in EDTA; (□) C1r alone in Ca^{2+} ; and (■) C1r+ Fab_2 in Ca^{2+} .

vation in a dose-dependent fashion, and had no effect on C1r activation in the absence of Ca^{2+} (not shown). A likely explanation for this potentiating effect of the anti-peptide antibodies is that they stabilize the C1r interaction region in a Ca^{2+} -bound conformation that enhances the inhibitory mechanism of Ca^{2+} . Although this effect of the antibodies allows no firm conclusion with respect to the precise role of the large loop of the EGF module in this mechanism, it provides direct support for the involvement of the EGF module in this process. Other data (not shown) indicated that the anti-peptide Fab_2 fragments, and peptide C1r (129–151) itself had no significant effect on the interaction between C1q and C1r or the C1s–C1r–C1r–C1s tetramer.

It is well-established that complete stabilization of C1r in the proenzyme form is achieved upon incorporation of the protein within the Ca^{2+} -dependent C1s–C1r–C1r–C1s tetramer, and the same effect can be obtained when C1s is replaced by its N-terminal α fragment, i.e., upon formation of the C1s α –

C1r–C1r–C1s α complex (Thielens et al., 1994). It appears therefore that interaction of the α region of C1r with the corresponding region of C1s enhances the stabilizing effect exerted by Ca^{2+} alone, thereby leading to complete inhibition of C1r autoactivation. In this respect, it may be hypothesized that the antibodies directed to the large loop of the C1r EGF module possibly mimic the effect exerted by C1s α . In any case, the occurrence of a Ca^{2+} -dependent regulatory mechanism implies transmission of a signal from the N-terminal α region of C1r to its C-terminal catalytic domain, either through the polypeptide backbone or through direct contact between these regions. The latter hypothesis appears plausible in light of studies providing evidence for interactions between distal domains in other modular serine proteases such as tissue plasminogen activator (Novokhatny et al., 1991) and protein C (Öhlin and Stenflo, 1987).

In contrast with the observed stability of proenzyme C1r within the tetramers C1s–C1r–C1r–C1s and C1s α –C1r–C1r–C1s α , it was shown that formation of complexes between either tetramers and C1q releases the Ca^{2+} -dependent inhibitory mechanism and allows C1r to fully recover its ability to activate (Thielens et al., 1994). If one considers that the major site of interaction between C1s–C1r–C1r–C1s and C1q very likely lies within the CUB–EGF moieties of C1r and C1s, it may be hypothesized that the interaction between these domains and C1q generates the signal that restores the activation potential of C1r within C1, and thereby triggers C1 activation (Thielens et al., 1994). Thus, the CUB–EGF module pairs of C1r and C1s not only are keystones of the C1 architecture, but may be considered as the interface between the recognition function of the complex, mediated by the globular domains of C1q, and its proteolytic function, mediated by the catalytic regions of C1r and C1s.

4. Conclusions and perspectives

Studies performed in the past few years, particularly the production of various recombinant modular fragments and their precise functional characterization, have allowed identification of the domains of

C1r and C1s that mediate their interaction properties. Available data provide clear evidence that the N-terminal CUB–EGF module pairs of both proteases mediate Ca^{2+} -dependent assembly of the C1s–C1r–C1r–C1s tetramer and provide sites for interaction between the tetramer and C1q. In addition to this essential structural role, there is growing evidence that the interaction region of C1r, most probably its CUB–EGF pair, plays a key part in the regulation of the C1r autoactivation property, and hence in the control of C1 activation.

These considerations underline the need to get deeper insights into the structure–function relationships of the CUB–EGF domains of C1r and C1s in order to identify the ligands and the molecular mechanisms involved in Ca^{2+} binding and protein–protein interaction. These objectives will require the use of X-ray crystallography and NMR spectroscopy to solve the three-dimensional structure of the domains, as well as the combined use of site-directed mutagenesis and functional studies to identify key amino acid residues and decipher interaction mechanisms at the atomic level. This approach will generate information that is essential for a better understanding of the assembly and function of the C1 complex. In addition, such information could be utilized for the rational design of synthetic compounds aimed at blocking C1 function and hence complement activation under circumstances where this leads to undesired or pathological effects.

Acknowledgements

This work was supported in part by the Commissariat à l'Energie Atomique, the Centre National de la Recherche Scientifique, and the European Union biotechnology programme (contract no. BIO 4 CT 960662). This is publication no. 569 from the Institut de Biologie Structurale.

References

- Arlaud, G.J., Villiers, C.L., Chesne, S., Colomb, M.G., 1980. Purified proenzyme C1r. Some characteristics of its activation and subsequent proteolytic cleavage. *Biochim. Biophys. Acta* 616, 116–129.
- Arlaud, G.J., Colomb, M.G., Gagnon, J., 1987. A functional model of the human C1 complex. *Immunol. Today* 8, 106–111.
- Arlaud, G.J., Volanakis, J.E., Thielens, N.M., Narayana, S.V.L., Rossi, V., Xu, Y., 1998. The atypical serine proteases of the complement system. *Adv. Immunol.* 69, 249–307.
- Bersch, B., Hernandez, J.-F., Marion, D., Arlaud, G.J., 1998. Solution structure of the epidermal growth factor (EGF)-like module of human complement protease C1r, an atypical member of the EGF family. *Biochemistry* 37, 1204–1214.
- Bork, P., Beckmann, G., 1993. The CUB domain. A widespread module in developmentally regulated proteins. *J. Mol. Biol.* 231, 539–545.
- Bork, P., Bairoch, A., 1995. Extracellular protein modules: a proposed nomenclature. *Trends Biochem. Sci.* 20, C03, Suppl. March.
- Busby, T.F., Ingham, K.C., 1987. Calcium-sensitive thermal transitions and domain structure of human complement subcomponent C1r. *Biochemistry* 26, 5564–5571.
- Busby, T.F., Ingham, K.C., 1988. Domain structure, stability, and interactions of human complement C1s: characterization of a derivative lacking most of the B chain. *Biochemistry* 27, 6127–6135.
- Busby, T.F., Ingham, K.C., 1990. NH_2 -terminal calcium-binding domain of human complement C1s mediates the interaction of C1r with C1q. *Biochemistry* 29, 4613–4618.
- Campbell, I.D., Bork, P., 1993. Epidermal growth factor-like modules. *Curr. Opin. Struct. Biol.* 3, 385–392.
- Cooper, 1985. The classical complement pathway: activation and regulation of the first complement component. *Adv. Immunol.* 37, 151–216.
- Cseh, S., Gal, P., Sarvari, M., Dobo, J., Lorincz, Z., Schumaker, V.N., Zavodszky, P., 1996. Functional effects of domain deletions in a multidomain serine protease, C1r. *Mol. Immunol.* 33, 351–359.
- Downing, A.K., Knott, V., Werner, J.M., Cardy, C.M., Campbell, I.D., Handford, P.A., 1996. Solution structure of a pair of calcium-binding epidermal growth factor-like domains: implications for the Marfan syndrome and other genetic disorders. *Cell* 85, 597–605.
- Harlow, E., Lane, D., 1988. *Antibodies. A laboratory manual.* Cold Spring Harbor Laboratory, pp. 626–631.
- Hernandez, J.-F., Bersch, B., Pétillot, Y., Gagnon, J., Arlaud, G.J., 1997. Chemical synthesis and characterization of the epidermal growth factor-like module of human complement protease C1r. *J. Peptide Res.* 49, 221–231.
- Illy, C., Thielens, N.M., Gagnon, J., Arlaud, G.J., 1991. Effect of lactoperoxidase-catalyzed iodination on the Ca^{2+} -dependent interactions of human C1s. Location of the iodination sites. *Biochemistry* 30, 7135–7141.
- Lacroix, M.B., Aude, C.A., Arlaud, G.J., Colomb, M.G., 1989. Isolation and functional characterization of the proenzyme form of the catalytic domains of human C1r. *Biochem. J.* 257, 885–891.
- Lakatos, S., 1987. Subunit interactions in the first component of complement, C1. *Biochem. Biophys. Res. Commun.* 149, 378–384.
- Novokhatny, V.V., Ingham, K.C., Medved, L.V., 1991. Domain

- structure and domain–domain interactions of recombinant tissue plasminogen activator. *J. Biol. Chem.* 266, 12994–13002.
- Öhlin, A.K., Stenflo, J., 1987. Calcium-dependent interaction between the epidermal growth factor precursor-like region of human protein C and a monoclonal antibody. *J. Biol. Chem.* 262, 13798–13804.
- Prahl, J.W., Porter, R.R., 1968. Allotype-related sequence variation of the heavy chain of rabbit immunoglobulin G. *Biochem. J.* 107, 753–763.
- Rao, Z., Handford, P.A., Mayhew, M., Knott, V., Brownlee, G.G., Stuart, D., 1995. The structure of a Ca^{2+} -binding epidermal growth factor-like domain: its role in protein–protein interactions. *Cell* 82, 131–141.
- Reid, K.B.M., Bentley, D.R., Campbell, D.R., Chung, L.P., Sim, R.B., Kristensen, T., Tack, B.F., 1986. Complement system proteins which interact with C3b or C4b. *Immunol. Today* 7, 230–234.
- Rivas, G., Ingham, K.C., Minton, A.P., 1992. Calcium-linked self-association of human complement C1s. *Biochemistry* 31, 11707–11712.
- Rivas, G., Ingham, K.C., Minton, A.P., 1994. Ca^{2+} -linked association of human complement C1s and C1r. *Biochemistry* 33, 2341–2348.
- Schumaker, V.N., Zavodszky, P., Poon, P.H., 1987. Activation of the first component of complement. *Annu. Rev. Immunol.* 5, 21–42.
- Siegel, R.C., Schumaker, V.N., 1983. Measurement of the association constants of the complexes formed between intact C1q or pepsin-treated C1q stalks and the inactivated or activated $\text{C1r}_2\text{C1s}_2$ tetramers. *Mol. Immunol.* 20, 53–66.
- Sunnerhagen, M., Olah, G.A., Stenflo, J., Forsen, S., Drakenberg, T., Trehwella, J., 1996. The relative orientation of Gla and EGF domains in coagulation factor X is altered by Ca^{2+} binding to the first EGF domain. A combined NMR-small angle X-ray scattering study. *Biochemistry* 35, 11547–11559.
- Tamura, T., Bauer, H., 1982. Monoclonal antibody against the carboxy terminal peptide of pp60^{src} of Rous sarcoma virus reacts with native pp60^{src}. *EMBO J.* 1, 1479–1485.
- Thielens, N.M., Aude, C.A., Lacroix, M.B., Gagnon, J., Arlaud, G.J., 1990a. Ca^{2+} binding properties and Ca^{2+} -dependent interactions of the NH_2 -terminal α fragments of human complement proteases C1r and C1s. *J. Biol. Chem.* 265, 14469–14475.
- Thielens, N.M., Van Dorsselaer, A., Gagnon, J., Arlaud, G.J., 1990b. Chemical and functional characterization of a fragment of C1s containing the epidermal growth factor homology region. *Biochemistry* 29, 3570–3578.
- Thielens, N.M., Illy, C., Bally, I.M., Arlaud, G.J., 1994. Activation of human complement serine protease C1r is down-regulated by a Ca^{2+} -dependent intramolecular control that is released in the C1 complex through a signal transmitted by C1q. *Biochem. J.* 301, 509–516.
- Thielens, N.M., Enrie, K., Lacroix, M., Jaquinod, M., Hernandez, J.-F., Esser, A.F., Arlaud, G.J., 1998. The N-terminal CUB–EGF module pair of human C1r binds Ca^{2+} with high affinity and mediates calcium-dependent interaction with C1s. *Mol. Immunol.* 35, 332, abstract.
- Tsai, S.-W., Poon, P.H., Schumaker, V.N., 1997. Expression and characterization of a 159 amino acid, N-terminal fragment of human complement component C1s. *Mol. Immunol.* 34, 1273–1280.
- Villiers, C.L., Arlaud, G.J., Painter, R.H., Colomb, M.G., 1980. Calcium binding properties of the C1 subcomponents C1q, C1r and C1s. *FEBS Lett.* 117, 289–294.
- Villiers, C.L., Chesne, S., Lacroix, M.B., Arlaud, G.J., Colomb, M.G., 1982. Structural features of the first component of human complement, C1, as revealed by surface iodination. *Biochem. J.* 203, 185–191.
- Villiers, C.L., Arlaud, G.J., Colomb, M.G., 1984. Diamine-induced dissociation of the first component of human complement, C1. *Eur. J. Biochem.* 140, 421–426.
- Villiers, C.L., Arlaud, G.J., Colomb, M.G., 1985. Domain structure and associated functions of subcomponents C1r and C1s of the first component of human complement. *Proc. Natl. Acad. Sci. USA* 82, 4477–4481.
- Zavodszky, P., Gal, P., Cseh, S., Schumaker, V.N., 1993. Protein engineering studies on C1r and C1s. *Behring Inst. Mitt.* 93, 103–114.
- Ziccardi, R.J., Cooper, N.R., 1976. Activation of C1r by proteolytic cleavage. *J. Immunol.* 116, 504–509.

STIC-ILL

349199

From: Romeo, David
Sent: Monday, June 04, 2001 11:46 AM
To: STIC-ILL
Subject: 09503421 Arlaud GJ, Thielens NM, Illy C.

3347158

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

Arlaud GJ, Thielens NM, Illy C.

Assembly of the C1 complex.

Behring Inst Mitt. 1993 Dec; (93):189-95.

PMID: 8172567

NO
6/4

Assembly of the C1 Complex

G. J. ARLAUD, N. M. THIELENS, C. ILLY

Laboratoire d'Enzymologie Moléculaire, Institut de Biologie Structurale, 41 avenue des Martyrs, 38027 Grenoble Cedex 1, France

Summary

The C1 complex of complement is a Ca^{2+} -dependent complex protease comprising two loosely interacting subunits. C1q, the recognition subunit, is a hexameric protein with six peripheral globular domains, each connected through collagen-like "arms" to a central fibril-like "stalk". The catalytic subunit, C1s-C1r-C1r-C1s, is a Ca^{2+} -dependent tetrameric association of two serine protease zymogens, C1r and C1s, that are sequentially activated by cleavage of a single peptide bond, upon binding of C1 to activators. Each monomeric protease is comprised of six structural motifs which form at least four domains, distributed in two functionally distinct regions, α (N-terminal) and γ -B (C-terminal). The catalytic (γ -B) regions of C1r and C1s are respectively located in the centre and at each end of the isolated tetramer, and the Ca^{2+} -dependent C1r-C1s associations are mediated by the interaction (α) regions, which contain one Ca^{2+} binding site each. Physicochemical and electron microscopy studies indicate that the tetramer, which is highly elongated, folds into a more compact conformation upon interaction with C1q. Various models for C1 have been proposed, in which the tetramer either interacts with the outside part of the C1q arms (O- and W-shaped models), or is folded within the C1q arms (S- or 8-shaped models). These models are discussed in light of available information and in consideration of the structural requirements of C1 activation and function.

Key-Words: C1, serine protease, domain structure, Ca^{2+} binding.

Introduction

It was shown thirty years ago that C1 is a Ca^{2+} -dependent macromolecular complex comprised of three types of proteins, C1q, C1r, and C1s (Lepow et al., 1963). Since then, a large number of studies dealing with the structure and function of the C1 subcomponents have been carried out (reviewed by Reid, 1983; Cooper, 1985; Schumaker et al., 1987; Arlaud and Thielens, 1993). It is clearly established that C1 comprises two subunits, C1q, which mediates binding of C1 to immune complexes and non-immune activators, and C1s-C1r-C1r-C1s, a Ca^{2+} -dependent tetramer containing two serine proteases that are sequentially activated when C1 binds to an activator. A structural model of C1q was proposed early on the basis of amino acid sequence, protease digestion and electron microscopy studies (Reid and Porter, 1976). This model was later

shown to be compatible with steric and energetic constraints at the atomic level (Kilchherr et al., 1985). The structure of the C1s-C1r-C1r-C1s tetramer, in contrast, has remained unsolved for a longer period of time, due to the lack of information about the structure of C1r and C1s. Elucidation of their primary structure and studies involving limited proteolysis, electron microscopy and microcalorimetry have led to substantial progress in the knowledge of the domain structure and associated functions of these proteases, providing a better understanding of the structure of the C1s-C1r-C1r-C1s tetramer. The purpose of this short overview is to summarize these advances, as well as available information relating to the association of C1s-C1r-C1r-C1s with C1q, and to discuss current hypotheses about the structure of the C1 complex. A

(1993) On the phylogeny of the genes and C-chains of complement component for publication.

gas, P. (1986) A conformational predict helices in integral membrane biophys. Acta 869, 197.

ter, R. R. (1976) Subunit composition component C1q of the first complement. Biochem. J. 155, 19.

) Molecular cloning and characterization DNA and gene coding for the onent C1q of the human complement 231, 729.

8) The complement system. In: Mo- (Hames, B. D. and Glover, D. M., IRL Press.

ry, A. J. (1990) Ig-binding domains of ay 11, 387.

Villadangas, J. A. & Lopez de Castro, in the repertoire of peptides bound to and to site-specific mutants inside and Exp. Med. 177, 613.

iyoshi, R., Kuroiwa, T., Mihara, K. & unctions of signal and signal-anchor mined by the balance between the nt and the N-terminal charge. Proc. A. 89, 16.

D. J. & Reid, K. B. M. (1991) Characterization of the genes encoding the A- human complement subcomponent 4, 481.

P. J. M. & Rose, J. K. (1988) Evidence of signal sequence insertion into the um. Proc. Natl. Acad. Sci. U.S.A. 85,

5) A new method for predicting signal tes. Nucl. Acids Res. 14, 4863.

erbrook-Smith, S. (1990) Carboximide nan C1q and rabbit C1q and rabbit ol. 27, 221.

z, A. T., Jiang, H. X. & Gewurz, H. im amyloid P component oligomers re classical complement pathway via 76–92 of the A-chain collagen-like renol. 150, 169.

The A chains are derived from part of the proenzymes, and possess mosaic-like structures: divided into five structural motifs, different pairs of internal motifs IV/V and a single copy of motifs I and III are homologous to motifs in the human morphogenetic protein UVS.2 from *Xenopus laevis*, and to protein u EGF (Arlaud and Thielens, 1990). Motifs IV and V are tandem amino acid residues, homologous to the element called "short control region" or "complement control protein" found, usually in mul-

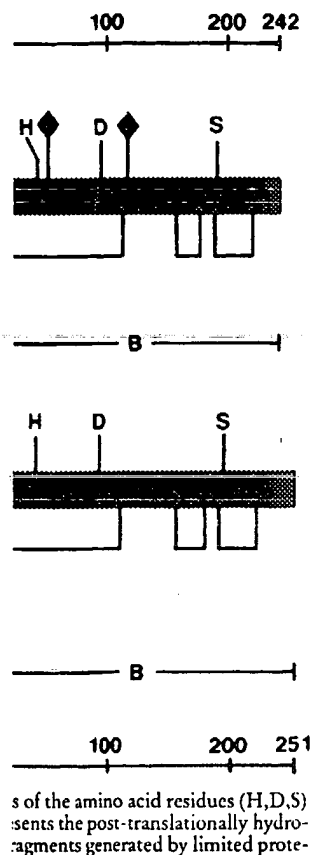
tiples, in a number of complement proteins sharing the ability to bind fragments C3b and/or C4b, and in other proteins apparently unrelated to the complement system (Reid et al., 1986). Motifs II are homologous to the Epidermal Growth Factor (EGF) and belong to a family of EGF-like domains found, e.g. in various blood coagulation factors and characterized by the presence of either a β -hydroxyaspartic acid or a β -hydroxyasparagine (Rees et al., 1988). The corresponding asparagine residue is hydroxylated fully in C1r, but only partially in C1s (Arlaud et al., 1987a; Thielens et al., 1990a).

Precise information on the domain structure of C1r and C1s was obtained in the past few years from electron microscopy, limited proteolysis, amino acid sequencing, differential scanning calorimetry and functional studies (reviewed by Arlaud and Thielens, 1993). Schematically, each monomeric protease is thought to comprise two functionally distinct regions (Fig. 1): (i) an interaction region (α) corresponding to the N-terminal half of the A chain, responsible for the Ca^{2+} -dependent C1r-C1s interactions involved in the assembly of the C1s-C1r-C1r-C1s tetramer; (ii) a catalytic region (γ -B) comprised of the C-terminal γ segment of the A chain and the B chain, responsible for the enzymatic reactions involved in the activation of C1 and the activity of C1.

The α regions of C1r and C1s exhibit characteristic low-temperature transitions (midpoints 32–37°C) that are abolished, or shifted to higher temperatures in the presence of Ca^{2+} ions (Busby and Ingham, 1987, 1988). Using limited proteolysis with trypsin under controlled conditions, fragments of about 200 amino acid residues corresponding to these regions of C1r and C1s have been obtained (Thielens et al., 1990b). These fragments, comprised of motifs I, II, and part of the following motif III (Fig. 1) each contain one Ca^{2+} binding site and retain the ability to mediate Ca^{2+} -dependent C1r-C1s heteroasso-

ciation. Various experiments (Thielens et al., 1990a; Illy et al., 1991) indicate that, in C1s, the structural determinants required for Ca^{2+} binding and Ca^{2+} -dependent C1r-C1s interaction are contributed by both the N-terminal motif I and the EGF-like motif II of the α region. In addition to its ability to mediate heterologous C1r-C1s association, fragment C1s α also retains the ability of native C1s to dimerize in the presence of Ca^{2+} , and this homologous (C1s-C1s) association provides one additional Ca^{2+} binding site (Thielens et al., 1990b).

A fragment corresponding to the catalytic γ -B region of C1s can be generated by limited proteolysis of C1s with plasmin (Villiers et al., 1985), and studies by differential scanning calorimetry (Medved et al., 1989) reveal that this region contains three independently folded domains, corresponding to motifs IV and V of the γ segment, and the B chain. This finding is consistent with neutron scattering experiments (Zaccari et al., 1990), and indicates that monomeric C1s, and probably also monomeric C1r, comprise at least four distinct domains: one or more in the α region, two in the γ region, and one in the B chain (Medved et al., 1989). In the case of C1r, autolytic cleavage, as well as limited proteolysis by extrinsic proteases of various specificities yield non-covalent (γ -B)₂ homodimers which represent the core of the native C1r-C1r molecule (Arlaud et al., 1986). The proenzyme form of these (γ -B)₂ regions has also been obtained by limited proteolysis, and was shown to contain all the structural elements that are necessary for intramolecular autoactivation (Lacroix et al., 1989). Neutron diffraction studies are consistent with a (γ -B)₂ dimer involving the loose packing of the two γ -B monomers (Zaccari et al., 1990), and chemical cross-linking experiments support the hypothesis of a "head to tail" configuration with heterologous interactions between the γ region of one monomer and the B chain of the other monomer (Arlaud et al., 1986). In contrast, electron microscopy



s of the amino acid residues (H,D,S) presents the post-translationally hydro-

after negative staining shows "asymmetric X"-shaped molecules compatible with homologous interactions between the γ regions and/or the B chains (Weiss et al., 1986; Perkins and Nealis, 1989).

Assembly of the C1s-C1r-C1r-C1s Tetramer

A number of studies based on ultracentrifugation, neutron scattering and electron microscopy indicate that the isolated C1s-C1r-C1r-C1s tetramer is highly elongated. By electron microscopy after negative staining (Tschopp et al., 1988), the tetramer appears as a linear array of 6-8 domains of unequal sizes, with an average width of 3-4 nm and a contour length of 51-59 nm, most of the molecules exhibiting a reversed "S" shape. Neutron scattering data obtained in solution are in agreement with these dimensions (Boyd et al., 1983). The above findings, and current knowledge of the domain structure of C1r and C1s are consistent with the model of the Ca^{2+} -dependent C1s-C1r-C1r-C1s tetramer shown in Figure 2. In this model (Villiers et al., 1985), the C1r-C1r dimer forms the core of the tetramer, with its catalytic (γ -B) regions in the centre, each of its interaction (α) regions being connected to the homologous region of a C1s molecule. The isolated C1s-C1r-C1r-C1s tetramer binds 4.0 Ca^{2+} atoms/mol, i.e. one Ca^{2+} atom per α region (Thielens et al., 1990b). That C1s forms the outer portion of the complex was demonstrated by affinity labelling experiments (Weiss et al., 1986). With respect to its overall shape and to the linear arrangement of the proteins and regions within the tetramer, the model shown in Figure 2 is still valid. However, it is schematic and obviously needs refinements with respect to the precise domain structure of C1r and C1s.

Assembly of the C1 Complex

Compared to the numerous studies performed on the individual C1 subcomponents, few studies dealing with the structure of macromolecular C1 have been carried out and most

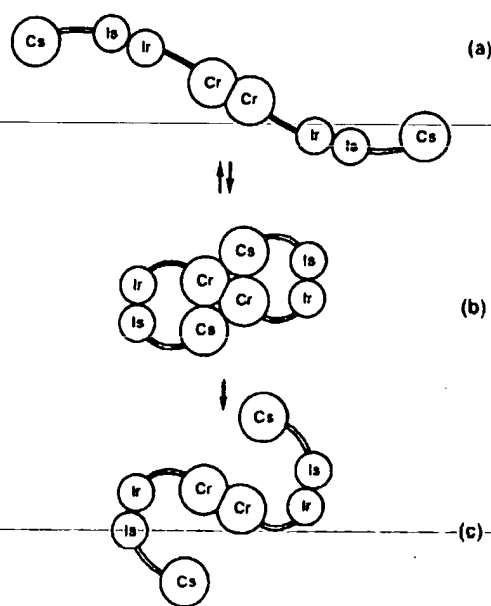


Fig. 2: Model of the Ca^{2+} -dependent C1s-C1r-C1r-C1s tetramer: (a) extended conformation; (b) compact "8"-shaped conformation; (c) open "S"-shaped conformation. Ir, Is: interaction (α) regions of C1r and C1s; Cr, Cs: catalytic (γ -B) regions of C1r and C1s.

of these have used C1 reconstituted from its purified subcomponents. C1 reassembled from C1q and the C1s-C1r-C1r-C1s tetramer has a sedimentation coefficient (15.9-16.3 S) and haemolytic activity comparable of those of C1 in serum (Ziccardi and Cooper, 1977; Siegel et al., 1981). At 4°C, the association constant for the interaction of proenzyme C1s-C1r-C1r-C1s with C1q is only $2-7 \times 10^7 \text{ M}^{-1}$, and it decreases by a factor of ten when the tetramer is activated (Siegel and Schumaker, 1983; Lakatos, 1987). Considering, however, that the interaction of C1q with the tetramer increases with increasing temperature (Ziccardi, 1985; Lakatos, 1987), it is likely that most, if not all, of proenzyme C1 is complexed under physiological conditions (Ziccardi and Cooper, 1978).

Comparative neutron scattering studies performed on C1q, the isolated C1s-C1r-C1r-C1s tetramer, and C1, strongly support the

hypothesis that the tetra compact conformation of C1q (Perkins et al., 1984) is located on the electron micrograph cross-linked C1 complex (Poon et al., 1983), in appears as a poorly defined centrally located on the the globular heads and top views, the extra mass is located inside the collagen arms. In agreement with al., 1977; Hsiung et al., 1983), electron provides evidence that the site for the interaction with C1 is located in the collagen molecule, probably in the

Available information suggesting of C1s-C1r-C1r-C1s process involving multiple steps contributed by both C1r and C1s enzyme C1r alone binds (Lakatos, 1987), but the forced by fragment C1s ability of the Ca^{2+} -dependent C1s tetramer to form a complex (Busby and Ingham, 1985) evidence (Villiers et al., 1985) suggest that the assembly part, ionic. This hypothesis reported by recent studies (Arlaud, manuscript in press) the involvement of a mechanism between basic (probably collagen-like region of C1s) acids of the tetramer, located

A structural and functional complex has been elaborated over years (Colomb et al., 1987b). This model was the respective locations of C1r and C1s in the center of the isolated tetramer (Figs. 1 and 2) that each of these regions

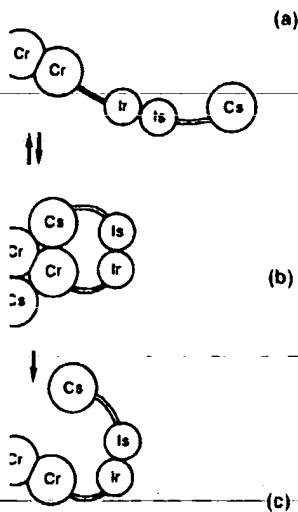


Fig. 2. Ca^{2+} -dependent C1s-C1r-C1r-C1s conformation; (b) compact "8"-shaped conformation; (c) open "S"-shaped conformation. Cr, Cs: catalytic regions of C1r and C1s; Ir, Is: catalytic regions of C1r and C1s.

C1 reconstituted from its components. C1 reassembled C1s-C1r-C1r-C1s tetramer on coefficient (15.9–16.3 S) activity comparable of those Ziccardi and Cooper, 1977; i. At 4°C, the association interaction of proenzyme with C1q is only 2–7 × raises by a factor of ten when activated (Siegel and Schumaker, 1987). Considering, how- cation of C1q with the tetra- h increasing temperature katos, 1987), it is likely that roenzyme C1 is complexed il conditions (Ziccardi and

on scattering studies per- the isolated C1s-C1r-C1r- C1, strongly support the

hypothesis that the tetramer folds into a more compact conformation upon interaction with C1q (Perkins et al., 1984). This can be visualized on electron micrographs of the chemically cross-linked C1 complex (Strang et al., 1982; Poon et al., 1983), in which the tetramer appears as a poorly defined but compact mass centrally located on the C1q arms, between the globular heads and the central stalk. On top views, the extra mass appears to be essentially located inside the cone defined by the C1q arms. In agreement with other data (Reid et al., 1977; Hsiung et al., 1988; Siegel and Schumaker, 1983), electron microscopy also provides evidence that the sites of C1q responsible for the interaction with C1s-C1r-C1r-C1s are located in the collagenous portion of the molecule, probably in the collagen-like arms.

Available information suggests that the binding of C1s-C1r-C1r-C1s to C1q is a complex process involving multiple sites that could be contributed by both C1r and C1s. Thus, proenzyme C1r alone binds weakly to C1q (Lakatos, 1987), but the interaction is reinforced by fragment C1s α , as shown by the ability of the Ca^{2+} -dependent C1s α -C1r-C1r-C1s α tetramer to form a pseudo-C1 complex (Busby and Ingham, 1990). Several lines of evidence (Villiers et al., 1984; Ziccardi, 1985) suggest that the assembly of C1 is, at least in part, ionic. This hypothesis is further supported by recent studies (Illy, Thielens, Arlaud, manuscript in preparation) indicating the involvement of a major ionic interaction between basic (probably lysine) residues of the collagen-like region of C1q, and acidic amino acids of the tetramer, located in C1r.

A structural and functional model of the C1 complex has been elaborated in the last few years (Colomb et al., 1984; Arlaud et al., 1987b). This model was originally based on the respective locations of the catalytic regions of C1r and C1s in the centre and at each end of the isolated tetramer (Fig. 2a). Considering that each of these regions contains both the

catalytic site and the Arg-Ile bond cleaved upon activation, it was proposed that, upon interaction with C1q, the tetramer folds into a compact "8"-shaped conformation (Fig. 2b), as this conformation brings the catalytic regions of C1r and C1s into the same vicinity, thereby allowing C1s activation by C1r. In the resulting C1 model, the tetramer is folded around two opposite C1q arms, and its overall shape is that of a distorted eight, with the α interaction regions lying outside the collagen-like arms of C1q, and the γ -B catalytic regions located inside the cone defined by the arms (Fig. 3). Once complete activation of C1 has occurred, then the tetramer could adopt a more relaxed "S"-shaped conformation (Fig. 2c) which could allow the catalytic regions of C1s to protrude outside the C1q molecule and thereby gain access to its substrates C4 and C2.

The models proposed by Schumaker et al. (1986), and by Weiss et al. (1986) are similar, although not strictly identical, to the above model. All three models, generally referred to

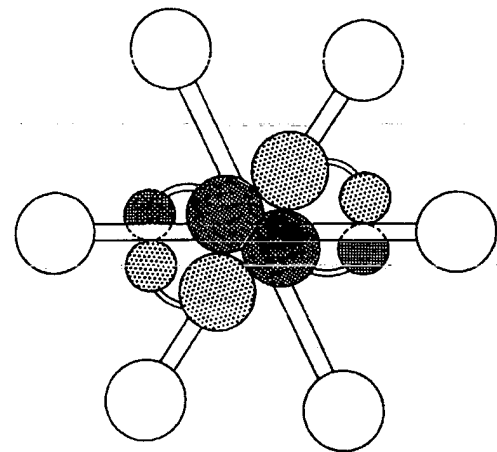


Fig. 3. Model of the C1 complex, viewed through the open end of the cone defined by the C1q arms. The C1s-C1r-C1r-C1s tetramer, with C1r squared and C1s dotted, is shown in the "8"-shaped conformation. Assembly of the complex is proposed to involve a major interaction between the interaction (α) regions of C1r and the collagen-like arms of C1q. Such an interaction is consistent with available information, but remains to be demonstrated.

as S-shaped or 8-shaped models, are compatible with the electron microscopy appearance of C1. They also address the structural requirements of C1 activation, and the concerns of symmetry inherent in the interaction between the tetramer, which has a 2-fold symmetry, and C1q, which exhibits a 3-fold symmetry (Poon et al., 1983). The major problem, however, about these models, is that it is difficult to visualize how the tetramer can readily dissociate from C1q if it is wrapped around and between the C1q arms. In the C- or O-shaped model proposed by Cooper (1985), the tetramer is first folded around its two-fold axis, its two C1r-C1s halves being stacked together, then wrapped in a ring-like fashion around the C1q stems. Perkins (1985, 1989) also considers various models on the basis of neutron scattering data, including a W-shaped model in which the tetramer binds to one side of the C1q stems. Both the C- and W-shaped models have the advantage of allowing unimpeded dissociation of C1s-C1r-C1r-C1s from C1q. However, they are not symmetrical, and do not appear to be fully consistent with electron micrographs which, as mentioned above, indicate that the major part of the tetramer is located inside the cone defined by the C1q stems.

Concluding Remarks

Substantial progress in the knowledge of the structure-function relationships of C1r and C1s has been made in the last few years, providing information that allows an overall picture of the C1s-C1r-C1r-C1s tetramer to be drawn. It seems to be clearly established, in particular from available physicochemical and electron microscopy data, that the tetramer folds into a compact conformation upon interaction with C1q, but the precise shape of the folded conformation remains to be determined. Among the various models that have been proposed for C1, the S- or 8-shaped models are generally considered the most plausible, but their validity is not proven.

Obviously, further progress in the knowledge of the structure and activation mechanism of C1 will require information about the three-dimensional structure and the precise function of the various domains or regions of each subcomponent, and identification of the complex protein-protein interactions involved in the assembly of C1.

References

- Arlaud, G. J., Gagnon, J., Villiers, C. L. & Colomb, M. G. (1986) Molecular characterization of the catalytic domains of human complement serine protease C1r. *Biochemistry* 25, 5177-5182.
- Arlaud, G. J., Van Dorsselaer, A., Bell, A., Mancini, M., Aude, C. A. & Gagnon, J. (1987a) Identification of erythro- β -hydroxyasparagine in the EGF-like domain of human C1r. *FEBS Lett.* 222, 129-134.
- Arlaud, G. J., Colomb, M. G. & Gagnon, J. (1987b). A functional model of the human C1 complex. *Immunol. Today* 8, 106-111.
- Arlaud, G. J. & Thielens, N. M. (1993) Human complement serine proteases C1r and C1s and their proenzymes. *Methods Enzymol.* 233, 61-82.
- Boyd, J., Burton, D. R., Perkins, S. J., Villiers, C. L., Dwek, R. A. & Arlaud, G. J. (1983) Neutron scattering studies of the isolated C1r2C1s2 subunit of first component of human complement in solution. *Proc. Natl. Acad. Sci. USA* 80, 3769-3773.
- Busby, T. F. & Ingham, K. C. (1987) Calcium-sensitive transitions and domain structure of human complement subcomponent C1r. *Biochemistry* 26, 5564-5571.
- Busby, T. F. & Ingham, K. C. (1988) Domain structure, stability, and interactions of human complement C1s: characterization of a derivative lacking most of the B chain. *Biochemistry* 27, 6127-6135.
- Busby, T. F. & Ingham, K. C. (1990) NH₂-terminal calcium-binding domain of human complement C1s mediates the interaction of C1r with C1q. *Biochemistry* 29, 4613-4618.
- Colomb, M. G., Arlaud, G. J. & Villiers, C. L. (1984) Activation of C1. *Phil. Trans. R. Soc. Lond. B* 306, 283-292.
- Cooper, N. R. (1985) The classical complement pathway: activation and regulation of the first complement component. *Adv. Immunol.* 37, 151-216.
- Hsiung, L. M., Dodds, A. W., Mason, D. W. & Reid, K. B. M. (1988) A monoclonal antibody to C1q which appears to interact with the C1r2C1s2-binding site. *FEBS Lett.* 229, 21-24.
- Illy, C., Thielens, N. M., Gagnon, J. & Arlaud, G. J. (1991) Effect of Lactoperoxidase-catalyzed iodination on the Ca²⁺-dependent interactions of human C1s. Location of the iodination sites. *Biochemistry* 30, 7135-7141.
- Kilchherr, E., Hofmann, H., (1985) A structural model of C1q comprising the link region of the six triple helices. *J. Mol. Biol.* 185, 885-891.
- Lacroix, M. B., Aude, C. A., Gagnon, J. & Arlaud, G. J. (1989) Isolation and functional characterization of the proenzyme form of the catalytic subcomponent C1r. *Biochem. J.* 257, 885-891.
- Lakatos, S. (1987) Subunit interaction of complement, C1. *Biochem. J.* 249, 378-384.
- Lepow, I. H., Naff, G. B., Todd, C. F. (1963) Chromatographic separation of human complement components. *J. Biol. Chem.* 238, 983-1008.
- Medved, L. V., Busby, T. F. & Ingham, K. C. (1989) Structural investigation of the domain structure of complement C1s: reversible assembly of the C1s subunit. *Biochemistry* 28, 5177-5182.
- Perkins, S. J. (1985) Molecular characterization of the subcomponent C1r2C1s2 derived from the first component of human complement. *Biochemistry* 24, 5177-5182.
- Perkins, S. J. (1989) Models of the C1 complex determined by physical techniques. *Immunol. Today* 10, 129-141.
- Perkins, S. J., Villiers, C. L., Arlaud, G. J., Colomb, M. G. & Burton, D. R. (1983) Neutron scattering studies of subcomponent C1 of human complement. *J. Mol. Biol.* 168, 563-577.
- Perkins, S. J. & Nealis, A. S. (1989) Structure in solution of human complement subcomponent C1r2C1s2. *Biochem. J.* 263, 461-468.
- Poon, P. H., Schumaker, V. N., & Arlaud, G. J. (1983) Conformation and reactivity of C1, the first component of complement. *Mol. Biol.* 168, 563-577.
- Rees, D. J. G., Jones, I. M., Esmouf, M. P., Smith, K. J. & Reid, K. B. M. (1983) The role of β -hydroxyaspartate residues in the first EGF domain of C1q. *J. Biol. Chem.* 258, 2053-2061.
- Reid, K. B. M. (1983) Proteins and control of the two pathways of complement. *Biochem. Soc. Trans.* 11, 1-12.
- Reid, K. B. M. (1989) C1q: gene and structure. *Behring Inst. Mitt.* 84, 8-12.
- Reid, K. B. M. & Porter, R. R. (1983) Structure and function of subcomponent C1r of complement. *Biochem. J.* 215, 1-12.
- Reid, K. B. M., Sim, R. B. & Porter, R. R. (1983) Structure of the haemolytic fragment of subcomponent C1s. *Biochem. J.* 215, 239-245.
- Reid, K. B. M., Bentley, D. R., L. P., Sim, R. B., Kristensen, T.

progress in the knowledge and activation mechanism of information about the structure and the precise function of each subunit or regions of each subunit identification of the complex interactions involved in the

J., Villiers, C. L. & Colomb, M. G. (1987) Identification of the catalytic domains of the serine protease C1r. *Biochemistry* 26, 5564-5571.

Dorselaer, A., Bell, A., Mancini, M., Gagnon, J. (1987a) Identification of the EGF-like domain of the first component of complement. *J. Biol. Chem.* 262, 129-134.

Colomb, M. G. & Gagnon, J. (1987b) A model for the human C1 complex. *Immunol. Rev.* 102, 129-141.

Thielens, N. M. (1993) Human complement C1r and C1s and their proenzymes. *Immunol. Rev.* 131, 61-82.

Reid, K. B., Perkins, S. J., Villiers, C. L., & Colomb, M. G. (1983) Neutron scattering studies of the C1r2C1s2 subunit of first component of complement in solution. *Proc. Natl. Acad. Sci. USA* 80, 73-77.

Reid, K. B. (1987) Calcium-sensitive structure of human complement C1r. *Biochemistry* 26, 5564-5571.

Reid, K. B. (1988) Domain structure of human complement C1s: a derivative lacking most of the B region. *J. Biol. Chem.* 263, 6127-6135.

Reid, K. B. (1990) NH₂-terminal catalytic site of human complement C1s mediated by C1q. *Biochemistry* 29, 151-156.

Reid, K. B. & Villiers, C. L. (1984) Activation of the first component of complement. *R. Soc. Lond. B* 306, 283-292.

The classical complement pathway: activation of the first component of complement. *Immunol. Rev.* 87, 151-216.

Reid, K. B., Mason, D. W. & Reid, K. B. (1987) A monoclonal antibody to C1q which appears to bind to the C1r2C1s2-binding site. *FEBS Lett.* 212, 1-5.

Reid, K. B., Gagnon, J. & Arlaud, G. J. (1991) Radiolabelled iodination of the amino groups of human C1s. Location of the amino groups. *Biochemistry* 30, 7135-7141.

Kilchherr, E., Hofmann, H., Steigemann, W. & Engel, J. (1985) A structural model of the collagen-like region of C1q comprising the link region and the fibre-like packing of the six triple helices. *J. Mol. Biol.* 186, 403-415.

Lacroix, M. B., Aude, C. A., Arlaud, G. J. & Colomb, M. G. (1989) Isolation and functional characterization of the proenzyme form of the catalytic domains of human C1r. *Biochem. J.* 257, 885-891.

Lakatos, S. (1987) Subunit interactions in the first component of complement, C1. *Biochem. Biophys. Res. Commun.* 149, 378-384.

Lepow, I. H., Naff, G. B., Todd, E. W., Pensky, J. & Hinz, C. F. (1963) Chromatographic resolution of the first component of human complement into three activities. *J. Exp. Med.* 117, 983-1008.

Medved, L. V., Busby, T. F. & Ingham, K. C. (1989) Calorimetric investigation of the domain structure of human complement C1s: reversible unfolding of the short consensus repeat units. *Biochemistry* 28, 5408-5414.

Perkins, S. J. (1985) Molecular modelling of human complement subcomponent C1q and its complex with C1r2C1s2 derived from neutron scattering curves and hydrodynamic properties. *Biochem. J.* 228, 13-26.

Perkins, S. J. (1989) Models for the C1 complex determined by physical techniques. *Behring Inst. Mitt.* 84, 129-141.

Perkins, S. J., Villiers, C. L., Arlaud, G. J., Boyd, J., Burton, D. R., Colomb, M. G. & Dwek, R. A. (1984) Neutron scattering studies of subcomponent C1q of first component C1 of human complement and its association with C1r2C1s2 within C1. *J. Mol. Biol.* 179, 547-557.

Perkins, S. J. & Nealis, A. S. (1989) The quaternary structure in solution of human complement subcomponent C1r2C1s2. *Biochem. J.* 263, 463-469.

Poon, P. H., Schumaker, V. N., Phillips, M. L. & Strang, C. J. (1983) Conformation and restricted segmental flexibility of C1, the first component of human complement. *J. Mol. Biol.* 168, 563-577.

Rees, D. J. G., Jones, I. M., Handford, P. A., Walter, S. J., Esnouf, M. P., Smith, K. J. & Brownlee, G. G. (1988) The role of β -hydroxyaspartate and adjacent carboxylate residues in the first EGF domain of human factor IX. *EMBO J.* 7, 2053-2061.

Reid, K. B. M. (1983) Proteins involved in the activation and control of the two pathways of human complement. *Biochem. Soc. Trans.* 11, 1-12.

Reid, K. B. M. (1989) C1q: genes, biosynthesis and biology. *Behring Inst. Mitt.* 84, 8-19.

Reid, K. B. M. & Porter, R. R. (1976) Subunit composition and structure of subcomponent C1q of the first component of complement. *Biochem. J.* 155, 19-23.

Reid, K. B. M., Sim, R. B. & Fairclough, A. P. (1977) Inhibition of the reconstitution of the haemolytic activity of the first component of human complement by a pepsin-derived fragment of subcomponent C1q. *Biochem. J.* 161, 239-245.

Reid, K. B. M., Bentley, D. R., Campbell, R. D., Chung, L. P., Sim, R. B., Kristensen, T. & Tack, B. F. (1986) Com-

plement system proteins which interact with C3b or C4b. *Immunol. Today* 7, 230-234.

Schumaker, V. N., Zavodszky, P. & Poon, P. H. (1987) Activation of the first component of complement. *Annu. Rev. Immunol.* 5, 21-42.

Siegel, R. C., Schumaker, V. N. & Poon, P. H. (1981) Stoichiometry and sedimentation properties of the complex formed between the C1q and C1r2C1s2 subcomponents of the first component of complement. *J. Immunol.* 127, 2447-2452.

Siegel, R. C. & Schumaker, V. N. (1983) Measurement of the association constants of the complexes formed between intact C1q or pepsin-treated C1q stalks and the unactivated or activated C1r2C1s2 tetramer. *Mol. Immunol.* 20, 53-66.

Strang, C. J., Siegel, R. C., Phillips, M. L., Poon, P. H. & Schumaker, V. N. (1982) Ultrastructure of the first component of human complement: electron microscopy of the crosslinked complex. *Proc. Natl. Acad. Sci. USA* 79, 586-590.

Thielens, N. M., Van Dorsselaer, A., Gagnon, J. & Arlaud, G. J. (1990a) Chemical and functional characterization of a fragment of C1s containing the Epidermal Growth Factor homology region. *Biochemistry* 29, 3570-3578.

Thielens, N. M., Aude, C. A., Lacroix, M. B., Gagnon, J. & Arlaud, G. J. (1990b) Ca²⁺ binding properties and Ca²⁺-dependent interactions of the isolated NH₂-terminal α fragments of human complement proteases C1r and C1s. *J. Biol. Chem.* 265, 14469-14475.

Tschopp, J., Villiger, W., Fuchs, H., Kilchherr, E. & Engel, J. (1980) Assembly of subcomponents C1r and C1s of first component of complement: electron microscopy and ultracentrifugal studies. *Proc. Natl. Acad. Sci. USA* 77, 7014-7018.

Villiers, C. L., Arlaud, G. J. & Colomb, M. G. (1984) Diamine-induced dissociation of the first component of human complement, C1. *Eur. J. Biochem.* 140, 421-426.

Villiers, C. L., Arlaud, G. J. & Colomb, M. G. (1984) Domain structure and associated functions of subcomponents C1r and C1s of the first component of human complement. *Proc. Natl. Acad. Sci. USA* 82, 4477-4481.

Weiss, V., Fauser, C. & Engel, J. (1986) Functional model of subcomponent C1 of human complement. *J. Mol. Biol.* 189, 573-581.

Zaccari, G., Aude, C. A., Thielens, N. M. & Arlaud, G. J. (1990) Neutron scattering study of the γ -B catalytic domains of complement proteases C1r and C1s. *FEBS Lett.* 269, 19-22.

Ziccardi, R. J. (1985) Nature of the interaction between the C1q and C1r2C1s2 subunits of the first component of human complement. *Mol. Immunol.* 22, 489-494.

Ziccardi, R. J. & Cooper, N. R. (1977) The subunit composition and sedimentation properties of human C1. *J. Immunol.* 118, 2047-2052.

Ziccardi, R. J. & Cooper, N. R. (1978) Direct demonstration and quantitation of the first complement component in human serum. *Science* 199, 1080-1082.

STIC-ILL

349197

From: Romeo, David
Sent: Monday, June 04, 2001 11:44 AM
To: STIC-ILL
Subject: 09503421 Day AJ, Barlow PN, Steinkasserer A, Campbell ID, Sim RB.

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

3347159

PLEASE COPY REFERENCE

Day AJ, Barlow PN, Steinkasserer A, Campbell ID, Sim RB.

Progress in determining module structures in C1r and C1s.

Behring Inst Mitt. 1993 Dec; (93):31-40.

PMID: 8172582

NO
6/4

Progress in Determining Module Structures in C1r and C1s

A. J. DAY, P. N. BARLOW, A. STEINKASSERER*, I. D. CAMPBELL, R. B. SIM*

Department of Biochemistry, and *MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U. K.

Correspondence to: Dr. R. B. Sim. MRC Immunochemistry Unit, Department of Biochemistry, South Parks Road, Oxford, OX1 3QU, U. K.

Key-Words: C1r, C1s, modules, tertiary structure, secondary structure.

Wiedemann, H., Timpl, R. & Reid, K. B. (1992) Binding of the pentamer/hexamer forms of IgG protein to zymosan activates the proenzyme complex, of the classical pathway of without involvement of C1q. *J. Immunol.*

1. C. & Reid, K. B. M. (1992) Purification, and cDNA cloning of human pulmonary surfactant protein A. *Biochem. J.* 284, 795-802.

Taurum, J., Thiel, S. & Sim, R. B. (1992) Invariant chain (C1qR) with Lung Surfactant Protein A. *Eur. J. Immunol.* 22, 1437-1445.

(1993) Collectin receptor (C1q receptor): function. *Behring Inst. Mitt.*, this volume.

Varki, A. & McEver, R. P. (1991) GMP-140: a protein receptor on human neutrophil: a lectin-like interaction. *J. Cell Biol.* 112,

Yada, M., Yamashina, I. & Kawasaki, T. (1984) Mechanism of carbohydrate-mediated complement activation by the serum mannan-binding protein. *Science* 225, 1980-1984.

M. S. & Drickamer, K. (1991) Determination of the carbohydrate-recognition domain in human lectins. *Glycobiology* 1, 615-21.

Morell, A. G. & Scheinberg, I. H. (1974) Human serum lectin. *Science* 186, 365-6.

Rezowska, K. & Drickamer, K. (1992) Complement binding by multiple carbohydrate domains in the macrophage mannose receptor. *EMBO J.* 11, 1719-1726.

) Mannan-binding protein, a complement activating lectin. *Immunopharmacol.* 24, 91-99.

Res, P., Rao, N., Foxall, C., Abbas, S., Das, M., Hasegawa, A., Kiso, M., Asa, D., Adley, B. K. (1991) Structural requirements for a carbohydrate ligand of E-selectin. *Proc. Natl. Acad. Sci. USA* 88, 10372-10376.

bn, R., Fourme, R., Drickamer, K. & Hendrickson, W. A. (1991) Structure of the calcium-dependent binding site of a rat mannose-binding protein determined by X-ray crystallography. *Science* 254, 1608-1615.

ickamer, K. & Hendrickson, W. A. (1992) Structure of the mannose-binding protein complexed with an oligosaccharide. *Nature* 360, 127-134.

The complement system proteases C1r and C1s are glycosylated proteins with highly elongated shapes. These characteristics make them unfavourable candidates for tertiary structure determination by X-ray crystallography. However, C1r and C1s are mosaic proteins, and are composed of several independently folding modules. These can be identified by searching, within the amino-acid sequences of C1r and C1s, for regions of repetitive sequence or for regions corresponding to consensus sequences of known modules (for review, see Arlaud et al., 1989; Fothergill et al., 1989). Early protein sequencing indicated that the B chains of activated C1r and C1s are serine protease domains, and analysis of the cDNA-derived amino acid sequences showed that the A chains of each contain five modules, namely one EGF (epidermal growth factor) module, two C1r/s modules and two CCP (complement control protein) modules (Fig. 1). Within the dumbbell shape of each C1r or C1s molecule, the serine protease domain corresponds to the globular region at one end, the two C1r/s and one EGF module are likely to form the globular mass at the other end, and the two CCP modules probably form the connecting strand (Fig. 1) (Perkins, 1989).

Information on the overall tertiary structure of C1r and C1s can therefore be obtained, not by crystallographic studies of the whole molecules, but by a segmental approach, deriving the tertiary structure of each type of module, then assembling these structures together to form a model of the whole protein. Individual modules can be synthesised as recombinant

proteins in eukaryotic or prokaryotic expression systems, and their tertiary structures determined by proton NMR (suitable for modules of less than approx. 20 kD), or by X-ray crystallography. Structures of modules can also be predicted from existing X-ray crystallographic or NMR data obtained from homologous modules. When tertiary structure data on each module are available, it may be possible to construct a model of the whole protein, provided that accurate information on the shape and flexibility of the protein in solution is available: this type of information is available for C1r and C1s from low angle x-ray and neutron solution scattering (Perkins and Nealis, 1989; Perkins, 1989; Zaccari et al., 1990).

At the Second International Workshop on C1 in 1988, modelling of the serine protease and EGF modules of C1r and C1s was presented (Fothergill et al., 1989), based respectively on the X-ray crystallographic structure of chymotrypsin (Cohen et al., 1981) and the NMR data of Cooke et al. (1987). Since that time, there has been rapid progress, presented below, in defining and characterising the other two module types, C1r/s and CCP.

The C1r/s Module Superfamily

C1r and C1s both contain two homologous sequences, about 110 amino acids in length, that flank their EGF modules (Leytus et al., 1986; Journet and Tosi, 1986; Mackinnon et al., 1987). This structure was originally detected only in these proteins and was called the "C1r/C1s specific repeat". Now it has been found in a large number of proteins, and will be referred

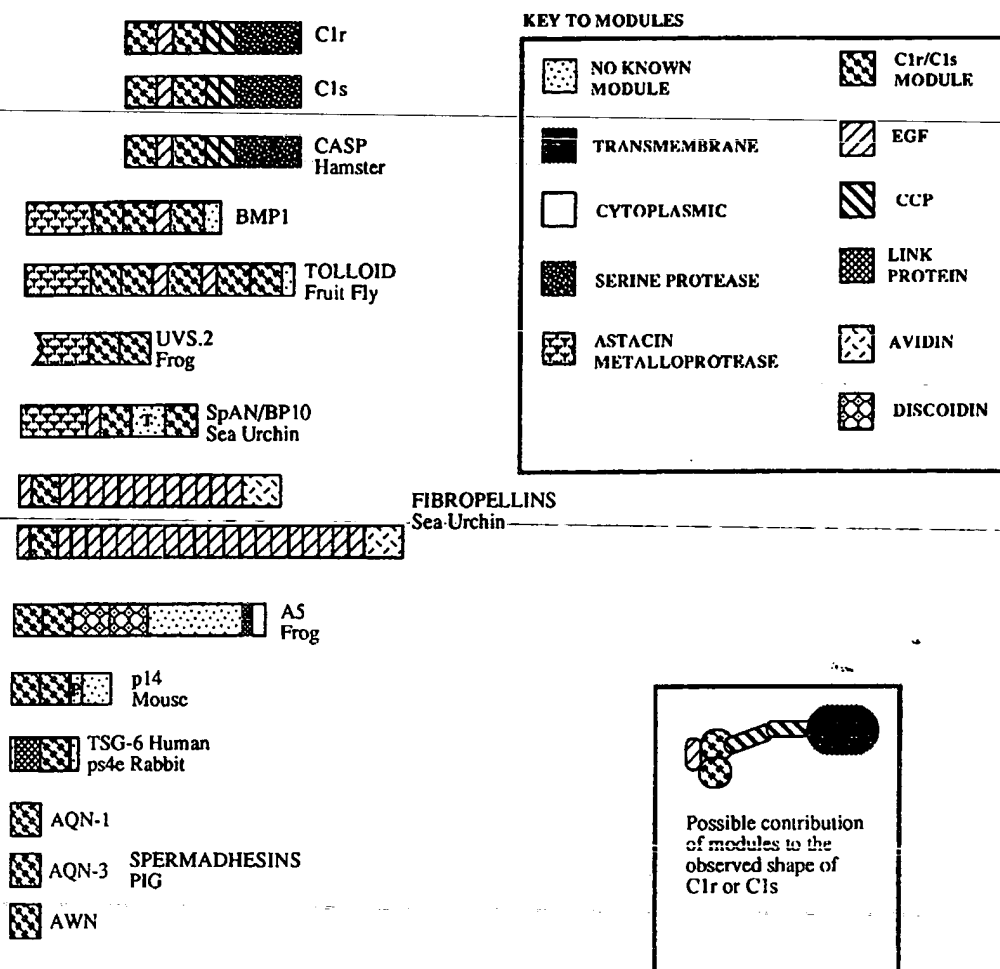


Fig. 1: The C1r/s module superfamily. The modular structure of proteins which contain the C1r/s domain is shown. The references for the proteins shown in this figure are given in the legend to Figure 2 except for purple sea urchin fibropellin [Delgadillo-Reynoso, M. G. et al., 1989, J. Mol. Evol. 29, 314-327], common sea urchin blastula protease-10 (BP10) [Gache, C. P., EMBL accession: S22060] and rabbit ps4e [Liau, G. et al., EMBL accession: M86381]. The modules and protein domains are defined on the figure. T and P represent regions that are threonine and proline rich, respectively. Only the C-terminal end of the UVS.2 protein has been sequenced and this is indicated by the incomplete astacin domain shown on the figure.

to as the C1r/s module. The characteristics of proteins containing C1r/s modules are shown in Figure 1, and comparison of the sequences of different C1r/s modules is shown in Figure 2, together with a consensus sequence.

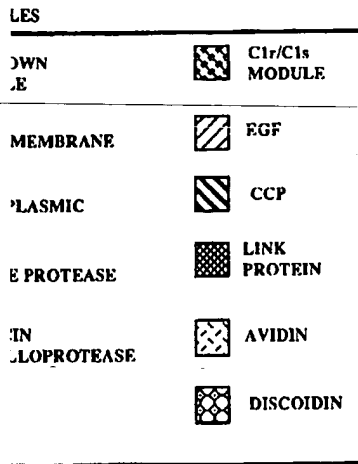
Calcium-dependent serine protease (CASP) from malignant hamster embryo fibroblasts

has an identical modular organisation to C1r and C1s (Fig. 1). CASP has greater than 80% sequence identity with human C1s and therefore CASP is likely to be the hamster analogue of C1s. CASP is thought to have a role in the degradation of extracellular matrix proteins including types I and IV collagen and fibronectin. Activated human C1s has also been

BMP1-2	ICGGDV	KDYCH	IQSPNY
TLD-2	VCCGDL	KLTKDQ	SDSPNY
BMP1-1	ACGCTL	QDSTGN	PSSPEY
BMP1-3	ACCGFL	TKLNGS	ITSPGN
P14-2	PCGGRM	EKAQCT	LTPPNM
P14-1	LCGGDV	TGESC	VASEGF
TLD-4	RCKPBI	TTSYGV	LQSPNY
SPAN-1	DCSYRP	TEMTGS	ITSPNY
TSG6	ECGGVP	TDPKRI	FKSPGF
TLD-5	ECGGVLRATNHSQ	TPYSHPRY	
TLD-3	ACCGVVDATKSNGS	LYSPSY	
A5PP-1	KCGDTI	KITSPSYLTSAGY	
UVS2-1	VCSMLL	PYSNGM	MISANY
A5PP-2	ECGRNP	TSSNGV	IKSPKY
UVS2-2	OCGGAP	YSSPKTTPSPNY	
SPAN-2	SCGCTP	VGVEGR	VASPNY
TLD-1	SCGRTY	QONSCH	IVSPHFIYSQ
CASP-2	NCSGNV	PTALTGR	ISSPNY
C1S-2	NCSGDV	PTALICE	IASPNY
C1R-2	ECSSSL	YTEASGY	TSRLEY
CASP-1	E	PTMHGE	ILSPNY
C1S-1	E	PTMYGE	ILSPNY
C1R-1	SIPIP	QKLPGE	VTSPLP
AQN3	DCGGPL	KNYSQW	ISY
AQN1	KCGGVL	RNYSGR	IST
AWN	SCGGVL	RDPCK	IFN
CON	CGG h	C I S P Y	

BMP1-2	HDSCAYDYLEVRDG	HGSEST
TLD-2	HDGCAYDFVEIRDG	NHSDGR
BMP1-1	SRLCYDYVEVRDG	PWRKAP
BMP1-3	NDVCYDFVEVRSG	LTADSK
P14-2	DTYCRYDSVSFNGCAVSDSK	
P14-1	HPSCRYDALEVFAG	SGTSCQ
TLD-4	HQECYDYVAIYDG	RSENS
SPAN-1	ETLCRYDAVEVRK	DDINS
TSG6	DPGCLADYVEIYDS	YDDVHG
TLD-5	SERCOYDYLEITSE	GYSMNT
TLD-3	TRPHYTKCYDYLYIYSMDNRK	
A5PP-1	RECKYDYVEIYDG	DNANQ
UVS2-1	SSOCVSDYIKIYDGPTKAPV	
A5PP-2	NAPOGQTCTRYDMLGIWDG	PPGVGPH
UVS2-2	GASCRYDYLNIYNS	TLGAV
SPAN-2	ETTCRWDSLMLNLC	NGIKV
TLD-1	SDDCTQDYLEIRDG	YWHKSP
CASP-2	DSQGNCG	DSLFLPAK
C1S-2	DSAGNCL	DSLVPVAG
C1R-2	QQVHCYPYDQLQIYAN	GKMK
CASP-1	SENCEYDSVQIISG	GVE
C1S-1	SENCAVDSVQIISG	DTF
C1R-1	SECCFYDYVKISAD	KK
AQN3	TCGKEYLEVRDQ	RACPD
AQN1	ACCKEYVEVQDC	LPAGH
AWN	SCCKEYVELLDG	PPGSE
CON	C YDYV I G	

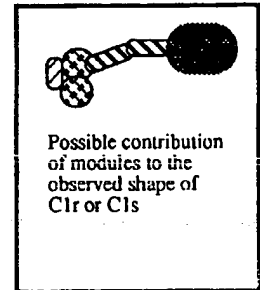
Fig. 2: C1r/s module multiple sequence alignment. The alignment was generated using the CLUSTAL program [Felsenstein, 1989]. A bias of 8 was added to the alignment. The alignment was performed for each pairwise comparison of sequences. The abbreviations used for the sequences are: BMP1-2 = BMP1-2; BMP1-1 = BMP1-1; BMP1-3 = BMP1-3; P14-2 = Mouse p14; P14-1 = Mouse p14; TLD-4 = TLD-4; SPAN-1 = SpAN; TSG6 = TSG-6; TLD-5 = TLD-5; TLD-3 = TLD-3; A5PP-1 = A5PP-1; UVS2-1 = UVS2-1; A5PP-2 = A5PP-2; UVS2-2 = UVS2-2; SPAN-2 = SPAN-2; TLD-1 = TLD-1; CASP-2 = CASP-2; C1S-2 = C1S-2; C1R-2 = C1R-2; CASP-1 = CASP-1; C1S-1 = C1S-1; C1R-1 = C1R-1; AQN3 = AQN3; AQN1 = AQN1; AWN = AWN; CON = Consensus sequence. An amino acid residue is indicated by an asterisk (*) and a dash (-) indicates a gap. The alignment is shown.



BMP1-2	ICQGDV	KKDYCH	IQSPNY	PDDY	RPSKVC	WRTQVSE	PHVGLTFQ	SFBIER
TLD-2	VCCGDL	KLTKDQ	SIDSPNY	PMDY	MPDKRC	WRTAPDN	HQVALKQF	SPELEK
BMP1-1	ACGETL	QDSTLN	PSSPEY	PNGY	SAMHIC	WRTISVTP	EKIIINPT	SLDLVR
BMP1-3	ACOCPL	TKLNGS	ITSPCN	PKSY	PPNKNCI	WQLVAPTQ	VRISLQPD	PFETEC
P14-2	PCGGRM	EKAQGT	LTPPMW	PESYYP	PGISCS	WHIAPSN	QVIMLTQF	KPDVEP
P14-1	LCGGDV	TGSGY	VASEGF	PNLY	PPNKKCI	WITITVPG	QTVSLSPR	VQDMEL
TLD-4	RCXFEI	TTSYGV	LQSPNY	PEDY	PRNIYCY	MHPQVLC	HRILQTFH	DFEVES
SPAN-1	DCGYRP	TEHTCE	ITSPHY	PSNY	EDMTAC	VYBIEGPV	GTIELTFL	DM81ST
TSG6	ECGQVP	TDPKRI	PKSPGF	PNEY	EDNQIC	YWHIRLKY	QRIHLSFL	DPDLSD
TLD-5	ECQGLR	ATYHSQ	TPYSHPRY	CSRPY	KRNHYC	WDRIQADPE	SSVKIRFL	HPRIEY
TLD-3	ACQGVV	DATKSNCS	LYSPSY	PDVY	PNSKQC	WNEVAPPN	HAVPLNFS	HPDLSD
A5PP-1	KCCDTI	KITSPSY	LTSACY	PHSY	PPSORCE	WLQAPENY	QRIHINFPN	HPDLSD
UVS2-1	VCSNLL	PYSNGM	MISANY	PSAY	PNNANC	CVMLIRTPSG	QVTLOQFQ	AFDIQS
A5PP-2	ECSSNP	TSSNGV	IKSPKY	PKSY	PNALECTY	IIPAPKM	QSVLEFE	SPELEADS
UVS2-2	QCQGAP	YSSPKT	ITSPNY	PCNY	TNTNCT	MTITAPAC	PKVSLRIT	DFELBI
SPAN-2	SCCGTF	VQVEGR	VASPNY	PNDY	DNSLQC	QDVYIEVDDG	RRVELIFE	DPGLBD
TLD-1	SCGRTY	QONSCH	IVSPHY	PSAESP	CDASTL	TNCEWRTATNG	EKVIHLQ	QHLMS
CASP-2	NCSCGV	PTALIGE	ISSPNY	PNPY	PENSRC	EQVILLERQ	POVVVT	QORDDPVEPA
C1S-2	NCSCDV	PTALICE	ISSPNY	PKPY	PENSRC	EQVIRLEKQ	POVVVT	LRREDFOVEAA
C1R-2	ECSSSEL	YTEASGY	ISSLEY	PRSY	PPDLRC	WYSIRVERC	LTMLHLKFL	EPPDIDDH
CASP-1	S	PTMHGE	ILSPNY	QOAY	PNEBKT	WDIEVPEG	PGVRLYPT	HLDMEL
C1S-1	E	PTMYGR	ILSPNY	QOAY	PSEVBKS	WDIEVPEG	YGLHLYPT	HLDIEL
C1R-1	SIPIP	QKLFGC	VTSPLP	PKPY	PNNFET	TTVTITVPTG	YRVKLVPQ	QPDLEP
AQN3	DCGGLF	KNYSCW	ISY	YKALT	TNCVMT	EMKPG	HKIIQL	PLNL
AQN1	KCCGVL	RNYSCR	IST	YEGPK	TDCTI	WTILAKPG	SRVPAIP	YLNL
AWN	SCCGVL	RDPPCK	IFN	SDCPQ	KDCVMT	IKVKPH	PHVLAIP	PLNL
CON	CCG h	G I S P Y		P Y P	C W I	G V L F	PDLE	

BMP1-2	HDSCAYD	LEVVRDC	HSESTL	IGRYCGY	EKPD	IKSTSSRL	WKFVSDGS	INK	ACPAVNFF
TLD-2	HDGCAYD	VEVIRDC	NHSDSRL	IGRPCGD	KLPPN	IKTRSNQMY	IRPVSDSSVQK		LCFSAALM
BMP1-1	SRLCAYD	VEVIRDC	FWRKAPLR	GRPCGS	KLPEP	IVSTDSRL	WVEFSSSNWVGK		GFFAVYEA
BMP1-3	NDVCYD	VEVIRDC	LTADSKLH	GKFCGS	SKPEV	ITSQYNNMR	VEFKSDNTVSKK		GPKAHFP
P14-2	DTYCRD	VSVFNGA	VSDDSKRL	GKFCGD	KAPSP	ISSSEGNEL	LQVPSDLSVTA		OGFSASRY
P14-1	HPSCRYD	ALEVFAG	SGTSGQRI	GRPCGT	FRAPV	VAPCNQVTL	RMTTDECTGCR		GFLWYS
TLD-4	HOECYD	YVAIYDC	RSENSSTL	GIYCGG	REPYA	VIASSTNEM	PHVLTADAGLQK		GPKATFV
SPAN-1	ETLCRYD	AVEVRK	DDINSIGEX	PCGN	TLPPV	QISSSQNMW	SFTSDPSITRR		GPKATYV
TSG6	DPGCLAD	YVEIYDS	YDDVHG	FGVRYCGD	ELPDD	IIISGNVMTL	KFLSDASVTA		GCPQIKYV
TLD-5	SERCYD	YLEITRE	GYSMTI	THGRPCGK	HKPPI	IIISNOSTLL	RAFQTDENSLR		GPAISPM
TLD-3	TRPHYTK	CNYDYL	IYSXMR	NRLKIGIYCGH	ELPPV	VNSQSILRL	EFYSDRTVQR		SGFVAKFV
A5PP-1	RECKYD	VEVIRDC	DNANGQLL	GKYCGK	IAPSP	LVSTGSPSI	PIRFVSDYBTPC		AGFSIRYE
UVS2-1	SSQCVSD	YIKIYD	GPPTKAP	PLVNRACGT	GLIPL	QIASTNQML	VEFVSDRAVTC		TGFKATYG
A5PP-2	NAPGQCT	CRYDMLC	IWDG	PCGVCPI	HGRYCCQ	NTPCRV	RSPTCILSMIFHTS	ATAK	ECFPANFS
UVS2-2	CASCYD	YLYNIYS	TLGAVM	GPYCGP	IDPHS	ATVSKSNM	MTMSDPSKQYK		GFSATYT
SPAN-2	ETTCRW	DSLMLNLG	NGIKVGM	KMCCR	EYPAAS	LVLSICNR	MELKLTGDSVNR		GFVASRY
TLD-1	SDDCTQ	DYLEIRDC	YHKSPL	VRRICGN	VSGEV	ITQTSMRL	LYNNRMAAKGY		RCPKARFE
CASP-2	DSQGNQ	DELLFAAK	NBPQ	PPCCGN	GPPCP	LIETHSN	LDIVPOTDLT	BOKK	GWKLRYH
C1S-2	DSAGNCL	DSLVEFVAG	DRQCP	GYCGH	GFPCPL	NIEKSNAL	DIIFOTDLTQKK		GWKLRYH
C1R-2	QQVHC	PYDQIYAN	GKNIG	EPCKG	QRPPD	LDTSSNAVD	LLFFTDSCGDSR		GWKLRYT
CASP-1	SENCYD	VSQIISG	GVEBCRL	CQRTSKNANS	PIVEFQI	PYNKLQV	IFRSDFSNEER	PTGFAAYYA	
C1S-1	SENCAYD	VSQIISG	DTEBCRL	CQRTSSNPHSP	IVERFQV	PYNKLQV	IPKSDPSNEER	PTGFAAYYV	
C1R-1	SECCPYD	VVKISAD	KKSLGR	PCQLGSP	LGPNPK	GFMSQGNKML	LTPHTDPSNEE		NCTIMFYK
AQN3	TCCKEY	LEVVRDC	RAGPDN	NPLKVC	CTGCV	YQSSXNVAT	VKYSRDSHHPA		SSFNYYFY
AQN1	ACGKEY	VEVQDG	LPGAGNY	GKLC	GIGLT	YQSSSNAL	SIKYSRTAGHSA		SSPDYIYY
AWN	SCCKEY	VELLDG	PPCSEI	ICKICG	GISLV	FRSSSNAT	IKRLRTSCHRA		SPPHYIYY
CON	C YDVI	I G	hg+hcg		P h S	N h h P	SD		+ GP h Y

Fig. 2: C1r/s module multiple sequence alignment. A multiple sequence alignment of members of C1r/s module superfamily. The alignment was generated using the AMPS programme [Barton, G. L., 1990. Methods Enzymol. 183, 403-428.] A bias of 8 was added to each term of the mutation data matrix and a break penalty of 6 was used. 100 random runs were performed for each pairwise comparison to allow calculation of mean random scores. The distances, in standard deviation units, of pairwise scores from mean random scores, for a particular comparison, were used to order the sequences. The abbreviations used for protein names are: A5PP = *Xenopus laevis* A5 protein precursor [Takagi, S. et al., 1991. Neuron 7, 295-307]; AQN1 = Boar spermadhesin AQN-1 [Calvete, J. J. et al., 1992. Eur. J. Biol. 205, 645-652]; AQN3 = Boar spermadhesin AQN-3 [Sanz, L. et al., 1991. FEBS Lett. 291, 33-36]; AWN = Boar spermadhesin AWN [Sanz, L. et al., 1992. Biochim. Biophys. Acta 1119, 127-132]; BMP1 = Human bone morphogenic protein-1 [Wozney, J. M. et al., 1988. Science 242, 1528-1534]; CASP = Hamster calcium-dependent serine protease [Kinoshita, H., 1989. FEBS Lett. 250, 411-415]; p14 = Mouse p14 [Lecain, E. et al., 1991. J. Neurochem. 56, 2133-2138]; TLD = Drosophila dorsal-ventral patterning gene tollid [Shimell, M. J. et al., 1991. Cell 67, 469-481]; TSG6 = Human tumour necrosis factor-inducible protein-6 [Lee, T. H. et al., 1992. J. Cell Biol. 116, 545-557]; SPAN = Purple sea urchin very early blastula stage protein SPAN [Reynolds, S. D. et al., 1992. Development 114, 769-786]; UVS2 = *Xenopus laevis* UVS.2 protein [Sato, S. M. and Sargent, T. D., 1990. Dev. Biol. 137, 135-141; EMBL accession: M27162]; CON = C1r/s module consensus sequence. An amino acid residue is included in the consensus if it is present in 50% of the sequences. h is used to denote an aliphatic hydrophobic residue and + is used where both arginine and lysine are present. The disulphide bond organisation is shown.



ich contain the C1r/s domain is shown. The re 2 except for purple sea urchin fibropellin sea urchin blastula protease-10 (BP10) [Ga-accession: M86381.] The modules and pro-leonine and proline rich, respectively. Only idicated by the incomplete astacin domain

tical modular organisation to C1r ig. 1). CASP has greater than 80% entity with human C1s and there-is likely to be the hamster analogue SP is thought to have a role in the of extracellular matrix proteins types I and IV collagen and fibro-ivated human C1s has also been

reported to degrade types I and II collagen (Yamaguchi et al., 1990; Sakiyama et al., 1991). In C1r, C1s and CASP an EGF domain is sandwiched between two C1r/s modules. Two other proteases contain such a C1r/s-EGF-C1r/s (CEC) cassette. They are human bone morphogenic protein-1 (BMP-1) and the fruit fly *tolloid* gene product (Fig. 1). BMP-1 is comprised of an N-terminal domain, found originally in a crayfish (*Astacus fluviatilis*) zinc metalloproteinase, a C1r/s module, a CEC cassette and a short non-homologous region. The *tolloid* gene product is very similar to BMP-1 but has an additional EGF and two contiguous C1r/s modules at its C-terminal end. BMP-1 and the *tolloid* gene product are also thought to have similar functions, where BMP-1 is involved in the formation of bone and cartilage in humans and *tolloid* regulates dorsal-ventral pattern formation in embryonic *Drosophila*. Both proteins are believed to mediate the binding to, and proteolytic processing of, TGF- β -related proteins (i.e., human BMP-2 and *Drosophila dpp* gene product), and thus take part in a growth-inducing signal transduction (Hecht and Anderson, 1992).

Three other C1r/s module superfamily members that contain an astacin metalloproteinase domain are involved in embryonic development, namely *Xenopus laevis* UVS.2, *Strongylocentrotus purpuratus* (purple urchin) SpAN and *Paracentrotus lividus* (common urchin) blastula protease-10 (BP10) where BP10 is probably a species analogue of SpAN. Their modular structures are shown on Figure 1. UVS.2 is involved in dorsal-ventral tissue differentiation in *Xenopus* embryos and SpAN in very early blastula stage development of the purple urchin animal-vegetal axis. Another embryonic urchin protein, fibropellin, contains a C1r/s module (Fig. 1). Fibropellin, which has two alternatively spliced forms with either 12 or 20 EGF domains, is a major component of the extracellular matrix (hyaline layer) forming a fibrous coat that surrounds

the embryo during development and is essential for holding the blastomeres together (Bisgrove et al., 1991). The C-terminal end of fibropellin is homologous to avidin and is serine and threonine-rich probably being heavily O-glycosylated. The discovery of the C1r/s module in echinoderms indicates this module evolved before invertebrate/vertebrate divergence (i.e., greater than 500 million years ago).

The A5 antigen from *Xenopus laevis* is a neuronal cell surface-protein that is thought to be involved in the neuronal recognition between optic nerve fibres and the visual centres during development. A5 comprises two N-terminal C1r/s modules followed by two discoidin modules (found in discoidin, a slime mold lectin, and coagulation factors V and VIII), a non-homologous domain, a transmembrane segment and a short intracytoplasmic sequence (Fig. 1). Mouge p14, expressed at high levels in adult mouse brain as well in a wide range of other adult tissues, also contains two N-terminal C1r/s modules and C-terminal non-homologous region (Fig. 1).

The human protein TSG-6 is secreted from fibroblasts in response to the inflammatory cytokines TNF α or IL-1 and consists of an N-terminal, hyaluronate-binding, link module and a C-terminal C1r/s module (Fig. 1). It seems likely that TSG-6 is involved in fibroblast adhesion and migration at sites of inflammation and wound repair. A likely role for the C1r/s module in this protein is carbohydrate and/or collagen binding. Databank searching with the TSG-6 sequence has identified a serum-inducible gene from rabbit smooth muscle cells which shows a high degree of identity with TSG-6 and is therefore likely to represent rabbit TSG-6.

Three proteins from wild boar sperm have been identified that consist solely of a single C1r/s module (Fig. 1), namely AQN-1, AQN-3 and zona pellucida-binding protein AWN (P. Bork, personal communication).

These proteins, which are on the sperm surface, are known to play an important role as receptors for carbohydrates on the oocyte zona pellucida during fertilization.

The C1r/s module is a sequence of 4 cysteine sulphide bonded in a 1-2 and cysteine 3-4 (Hess et al., 1991). AQN-1 and AQN-3 are in the first C1r/s module. CASP are the first two C1r/s modules. Figure 2, a multiple sequence alignment of C1r/s modules is shown. It indicates that there are many highly conserved residues. At present there are no data on this protein module determining structure from C1r or C1s and this is being pursued in several laboratories. Primary structure predictions of C1r/s modules from (Hess et al., 1989) indicates that the structure with β strand content of α helix.

In C1r and C1s the full length of the module is partially conserved. Domains of C1r and C1s binding consensus with EGF modules (Handford et al., 1991) abundant evidence that C1r and C1s bind calcium ions stabilise the module. The EGF module contains two C1r/s modules, for one end of the dimer (Fig. 1). This region of the Ca^{2+} -dependent C1r/C1s dimerisation), and C1s₂ complex with C1r (Medved et al., 1989; Thomsen et al., 1991). It seems likely that the C1r and C1s mediate their collagen "stalks" of C1q (carbohydrate attached to the

during development and is essential for the blastomeres together (Bischoff, 1991). The C-terminal end of the protein is homologous to avidin and is rich in threonine-rich probably being glycosylated. The discovery of the role in echinoderms indicates this role evolved before invertebrate/vertebrate (i. e., greater than 500 million years

ago). A protein from *Xenopus laevis* is a neurite outgrowth factor that is thought to be involved in the neuronal recognition between neurites and the visual centres during development. A5 comprises two N-terminal EGF-like modules followed by two discoidin-like modules. In discoidin, a slime mold lectin, a discoidin domain, a transmembrane domain and a short intracytoplasmic tail (Fig. 1). Mouse p14, expressed at the adult mouse brain as well as in a number of other adult tissues, also contains two N-terminal C1r/s modules and C-terminal discoidin-like region (Fig. 1).

The protein TSG-6 is secreted from chondrocytes in response to the inflammatory cytokines TNF α or IL-1 and consists of an N-terminal EGF-like module, a link module, a C-terminal C1r/s module (Fig. 1). It is thought that TSG-6 is involved in fibroblast migration and migration at sites of inflammation and wound repair. A likely role for the protein in this protein is carbohydrate binding. Databank searching for TSG-6 sequence has identified a cDNA which shows a high degree of similarity to TSG-6 and is therefore likely to be the TSG-6.

Proteins from wild boar sperm have been identified that consist solely of a single EGF-like module (Fig. 1), namely AQN-1, a zona pellucida-binding protein (Bork, personal communication).

These proteins, which are associated with the sperm surface, are known as spermadhesins and play an important role as counter-receptors for carbohydrate structures attached to the oocyte zona pellucida glycoproteins during fertilization.

The C1r/s module is based on a consensus sequence of 4 cysteines (Fig. 2) which are disulphide bonded in a pattern of cysteine 1-2 and cysteine 3-4 (Hess et al., 1991; see refs. for AQN-1 and AQN-3 in legend to Fig. 2). Only in the first C1r/s module of C1r, C1s and CASP are the first two cysteines missing. In Figure 2, a multiple sequence alignment of 26 C1r/s modules is shown, and it can be seen that there are many highly conserved residues. At present there are no tertiary structure data on this protein module, although work on determining structures of C1r/s modules, from C1r or C1s and from other proteins, is being pursued in several laboratories. Secondary structure prediction based only on the C1r/s modules from C1r and C1s (Perkins, 1989) indicates that the module has a globular structure with β strands and turns, and a low content of α helix.

In C1r and C1s the function of the individual modules is partially established. The EGF domains of C1r and C1s contain a Ca^{2+} ion-binding consensus which is found in many EGF modules (Handford et al., 1991). There is abundant evidence that the EGF modules in C1r and C1s bind calcium ions, and that calcium ions stabilise the conformation of this module. The EGF module, together with the two C1r/s modules, form a globular mass at one end of the dumbbell shape of C1r or C1s (Fig. 1). This region of the molecule mediates the Ca^{2+} -dependent C1s-C1r interaction (and C1s dimerisation), and interaction of the C1r₂C1s₂ complex with C1q (Arlaud et al., 1989; Medved et al., 1989; Thielens, et al., 1990 a, b). It seems likely that the C1r/s modules in C1r and C1s mediate their interaction with the collagen "stalks" of C1q (or possibly with carbohydrate attached to the collagen) and probably

also play a role, along with the EGF domain, in the formation of the tetrameric complex.

For most of the other members of the C1r/s superfamily there are little or no data to suggest what the role of this module is. In the case of the spermadhesins which consist of a single C1r/s module it seems clear that it is performing a lectin function. Although the spermadhesins are the most distantly related members of the C1r/s subfamily, this carbohydrate-binding function may be a general feature. For example, in sea urchin fibropellin carbohydrate binding of the N-terminal C1r/s module to the glycosylated avidin domain of another fibropellin molecule would allow head-to-tail association. If the avidin tail could bind several C1r/s-modules-at-once (3, for example) coupled with the existence of two forms of fibropellin, with different lengths of spacer arm (12 or 20 EGF modules), this would allow the construction of a complex but geometrically regular fibrous coat.

The C1r/s module is found in a number of proteins that are involved in developmental processes. In these sugar binding may or may not be involved in their function. The C1r/s module is probably responsible for a wide range of protein and carbohydrate interaction and this will become clearer as more C1r/s superfamily proteins are identified and more functional data becomes available.

The CCP Modules

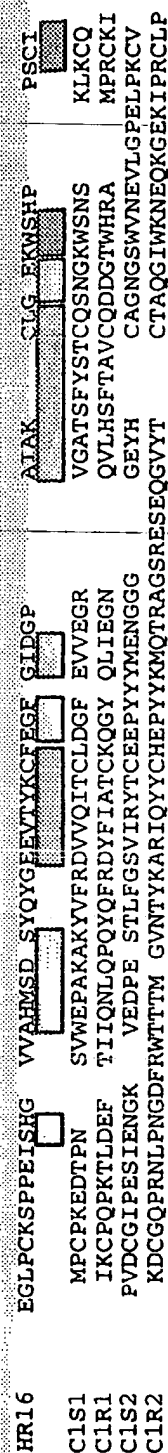
The nomenclature of the remaining module type is confusing, as it has been referred to as an SCR (short consensus repeat or short complement repeat), a B-type module (from its first observation in factor B), a C-module (denoting the association with complement), a sushi domain and a CCP (complement control protein repeat). Rapid progress in module characterisation is likely to generate a systematic nomenclature within a few years. This common module type, of which more than 150 examples are known, occurs in many comple-

ment proteins, including the control proteins Factor H, CR1, C4bp, DAF and MCP, in the receptor CR2, in the proteases C1r, C1s, Factor B and C2, and in the terminal components C6 and C7. The same module type also occurs in many non-complement proteins, such as the selectins, coagulation factor XIII b chain, β 2 glycoprotein 1, and IL-2 receptor (Reid and Day, 1989; Sim and Perkins, 1989). Within the complement proteins, the occurrence of this module type is associated with binding to one of the three homologous proteins, C3, C4 or C5. In C1s, there is evidence that one or both of the CCP domains is involved in interaction with the C1s substrate, C4 (Matsumoto et al., 1989).

Rapid progress has been made in determining tertiary structures of CCP modules (Barlow et al., 1991, 1992, 1993; Norman et al., 1991). CCP modules from complement factor H, which is made up of 20 contiguous CCP modules, have been synthesised in a yeast expression system, and the tertiary structures of three single modules (H-16, H-5 and H-15) have been determined by 2D-NMR. The structure of a double module (H-15 + H-16) has also been examined to establish how modules interact with each other. Comparison of the three single modules shows that all three have a highly conserved tertiary structure, although the extent of amino acid sequence identity between them is only 30–40%. H-15 and H-16, for example, are respectively 61–62 and 58–59 residues long, and have only 22 amino acid identities (Barlow et al., 1993). Very similar globular structures were observed in all three cases, with a hydrophobic core wrapped in β strands. A ribbon diagram representation of the tertiary structure is shown in Figure 3. The N- and C-termini are at opposite poles of the long axis of the molecule, and the two disulphide bridges, formed between cysteines 1–3 and 2–4, are close to the N- and C-terminus, respectively. Variations in sequence length are accommodated in a number of external loops (Fig. 3). In comparison to

many different CCP modules, one region is seen to be particularly variable in length and sequence, and this is accommodated in a "hypervariable" loop (Fig. 3). The conservation of core tertiary structure indicates that it is feasible to model the core structures of all CCP domains on the basis of the existing experimental structures, and this has been done, for example, for the fifth CCP domain of β 2 glycoprotein 1 (Steinkasserer et al., 1992). Modelling of longer external loops of variable sequence, however, may be inaccurate. A multiple sequence alignment of 150 CCP modules has been used to produce the alignment of the CCP modules of C1r and C1s with the sequence of H-16 (Fig. 3). The multiple sequence comparison increases the reliability with which segments of the C1r and C1s sequences can be assigned to loop or turn regions of the experimentally-determined tertiary structures.

C1r and C1s each contain two contiguous CCP modules. Recent studies of the double domain H-15 + H-16 in factor H (Barlow et al., 1993) show how CCP modules are connected to each other, and give information on the likely degree of flexibility (bending and rotation) between the modules in C1r and C1s. Medved et al. (1989) have shown in calorimetric studies that the two CCP modules of C1s unfold independently: consistent with this, in the H-15 + H-16 recombinant protein, the two modules fold autonomously, and there is only a small area of contact between the two modules (Fig. 4). This area of contact is made up from portions of the side chains of Phe 40 in H-15, and Tyr 25 in H-16, with contributions from His 13 and Arg 41 in H-15, and Leu 3 in H-16 (numbers for H-16 correspond to Fig. 3: in Fig. 4 the corresponding numbers for H-16 residues are Leu 64 and Tyr 86). The aromatic residues contributing to the area of contact are conserved in the C1r and C1s CCP modules (corresponding to Tyr 25 and Phe 37 in H-16, Fig. 3). The contact between the two modules limits the angle between their long axes, such



ent CCP modules, one region is particularly variable in length and this is accommodated in a "ble" loop (Fig. 3). The conservative tertiary structure indicates that it is model the core structures of all ins on the basis of the existing structural structures, and this has been ample, for the fifth CCP domain of stein 1 (Steinkasserer et al., 1992) of longer external loops of variable however, may be inaccurate. A multiple alignment of 150 CCP modules led to produce the alignment of the residues of C1r and C1s with the of H-16 (Fig. 3). The multiple comparison increases the reliability segments of the C1r and C1s can be assigned to loop or turn re-experimentally-determined tertiary.

1s each contain two contiguous les. Recent studies of the double 5 + H-16 in factor H (Barlow et show how CCP modules are to each other, and give information degree of flexibility (bending and between the modules in C1r and C1s. l. (1989) have shown in calorimetry that the two CCP modules of C1s identically: consistent with this, in H-16 recombinant protein, the two i autonomously, and there is only a of contact between the two g. 4). This area of contact is made ions of the side chains of Phe 40 in yr 25 in H-16, with contributions and Arg 41 in H-15, and Leu 3 in ers for H-16 correspond to Fig. 3: corresponding numbers for H-16 Leu 64 and Tyr 86). The aromatic tributing to the area of contact are i the C1r and C1s CCP modules ing to Tyr 25 and Phe 37 in H-16, contact between the two modules gle between their long axes, such

HR16	EGLPCKSPPEISHG	VVAHMSD	SYQVGEETVKCFEGF	GIDGP	AIAK	CLG	EKWSHP	PSCI
C1S1	MPCPKEDTPN	SVWEPAKAYVERDVQITCLDGF	EVVEGR		VGATSFYSTCQSNKGKWSNS			KLKCO
C1R1	IKCPQPKTLDEF	TIIONLPQYQFRDYFIATCKQGY	QLIEGN		QVLHSFTAVCQDDGTHRA			MPRCKI
C1S2	PVDCGIPESIENGK	VEDPE	STLFGSVIRYTCPEPYVMENG		GEYH			CAGNSWNEVLGPFLPKCV
C1R2	KDCGQPRNLPNGDFRWTTM	GVNTYKARIQYCYCHEPYKMKTRAGSRESEQGYT			CTAQGIWKNEQGEKIPRCCLP			

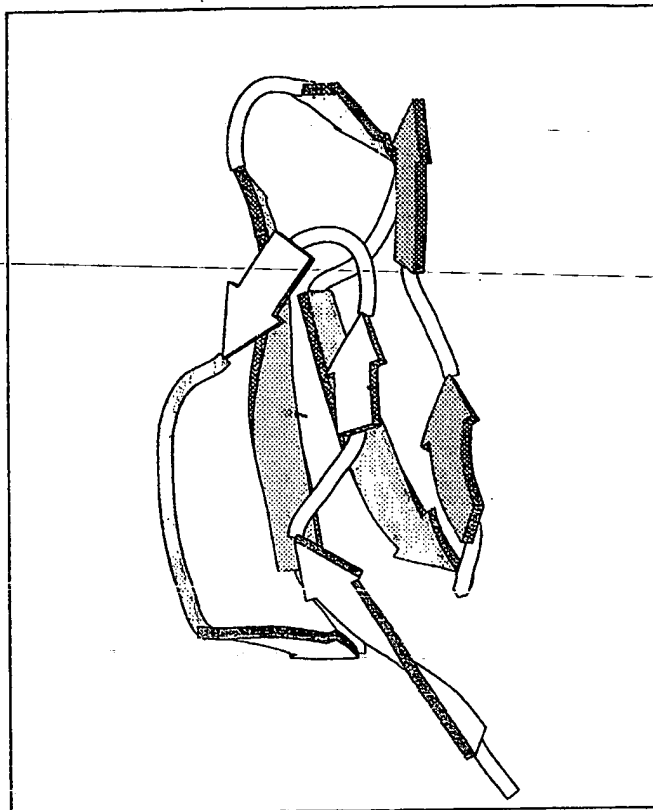


Fig. 3: Tertiary structure of CCP modules. A ribbon diagram of the tertiary structure of H-16 is shown. The N-terminus is at the left, and the C-terminus at the right. Broad segments with arrows indicate the β strands, and narrow segments indicate loops and areas of non-standard secondary structure. The upper (pink) loop is the "hypervariable" loop. When comparing different CCP modules, this is the region in which amino acid sequence is most variable in composition and length. The colour coding of tertiary structure regions corresponds to the segments of H-16 sequence shown at the top. The sequences of the CCP modules in C1r and C1s have been aligned to that of H-16 by multiple sequence alignment, as noted in the text.

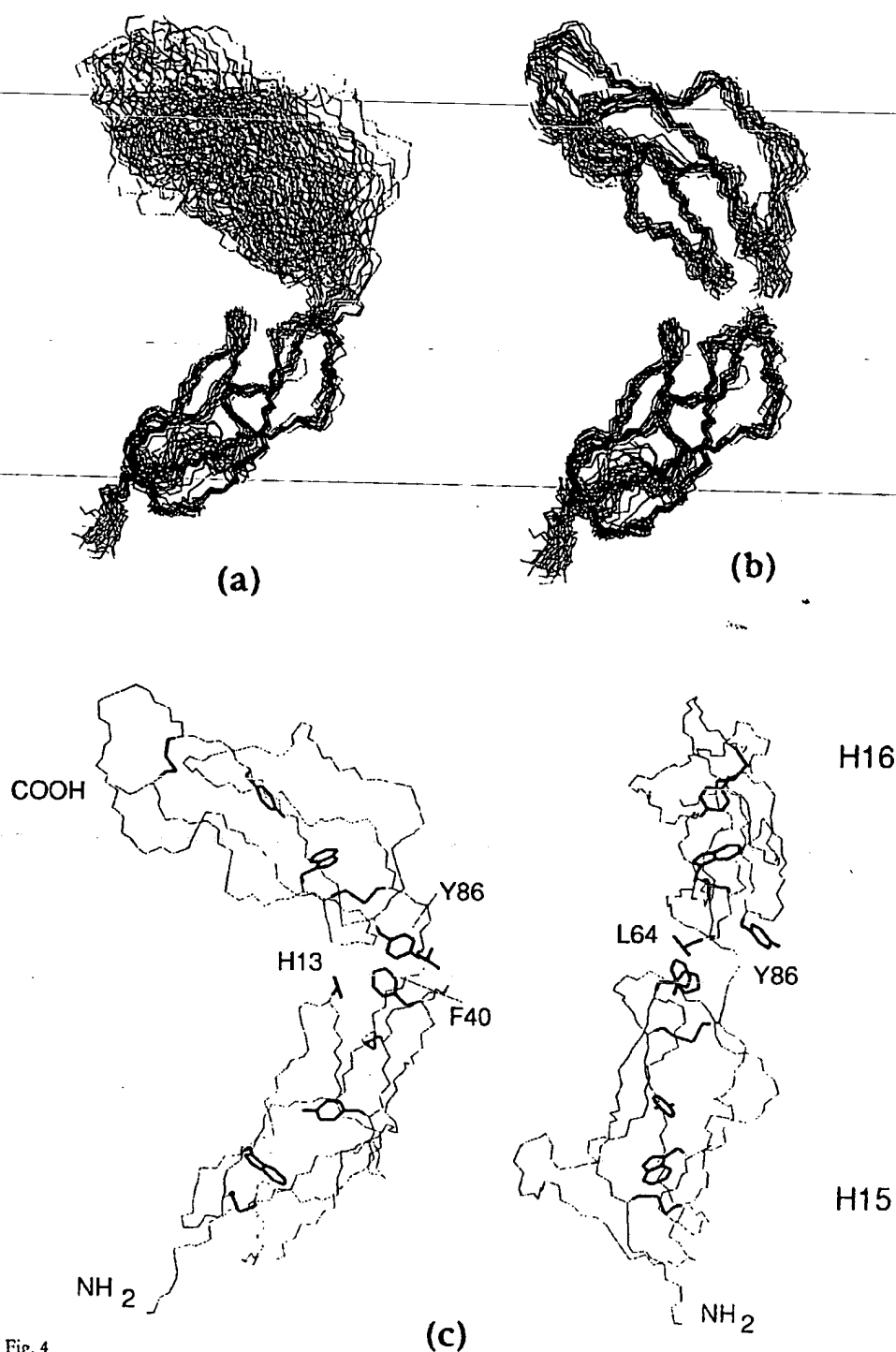


Fig. 4

Fig. 4: Structures for H15–16. (a) Backbone traces for 27 complementary C1r modules (residues 2–20 and 26–62). (b) A module-by-module superposition of 27 structures onto the equivalent of the average structure. This shows that each module is a similar structure. (c) Backbone atoms of the average structure. The two Trp residues, Tyr34, Tyr92, form a hydrophobic pocket at the interface not corresponding to the H-16.

that they take up a tilt or rotation around the long axis, only moderately restricted.

The data represented in Figure 4 (Barlow et al. (1991, 1992)) are used for modelling of the C1r/C1s, and from Figure 4 and comparison with the data for the C1r/C1s, it can be ascertained which module is hypervariable loop, and which is surface-exposed. This is the basis for manipulation of the modules, by site-directed mutagenesis, development of antibody libraries. Within a short time, data for the C1r/C1s module is available, and modelling of the molecules can be attempted.

Acknowledgements

A. J. Day is supported by the Rheumatism Council (UK). Barlow, A. Steinkasserer, and I. D. Campbell work within the Oxford Centre for Molecular Sciences (OCMS).

References

- Arlaud, G. J., Thielens, N. M. (1988) Structure and function of C1r. *Behring Inst. Mitt.* 84, 56–64.
- Barlow, P. N., Baron, M., Norman, D., Sim, R. B. & Campbell, I. D. (1991) Structure of the complement C1r/C1s two-dimensional ¹H-NMR.
- Barlow, P. N., Norman, D., Sim, R. B., Pearce, J., Driscoll, P. C., Sim, R. B. (1992) Solution structure of the complement C1r/C1s second example of the complement C1r/C1s module. *Biochemistry* 31, 36–41.



(b)

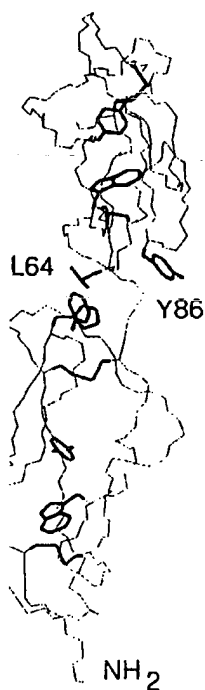


Fig. 4: Structures for H 15–16 calculated from nmr spectra.

(a) Backbone traces for 27 calculated structures after superposition of the H-15 component (i. e. using C α , C and N of residues 2–20 and 26–62). This illustrates that the relative orientation of the two modules is not precisely defined by the data.

(b) A module-by-module superposition (i. e. using C α , C and N of residues 2–20 and 20–62, or 63–78 and 84–119), for 27 structures onto the equivalent atoms of the minimised average structure (not drawn). Residue 63 is omitted for clarity. This shows that each module is well defined by the data.

(c) Backbone atoms of the average structure (two different rotations) shown by a thin line with side chains of the 8 Cys and 2 Trp residues, Tyr34, Tyr92, His13, Leu64, Phe40 and Tyr86. Some of the residues thought to contribute to the hydrophobic pocket at the interface are labelled. Note in this figure residue numbering is from the beginning of H-15, and does not correspond to the H-16 numbering in Figure 3. See text for details.

that they take up a tilted orientation. Relative rotation around the long axes of the modules is only moderately restricted.

The data represented in Figures 3 and 4 and in Barlow et al. (1991, 1992, 1993) provide a basis for modelling of the CCP modules of C1r and C1s, and from Figure 3, by simple inspection and comparison with the published data, it can be ascertained which residues are on the hypervariable loop, and which are internal or surface-exposed. This information provides a basis for manipulation of the activities of the modules, by site-directed mutagenesis or development of antibodies against surface loops. Within a short time, tertiary structure data for the C1r/s module will also become available, and modelling of the entire C1r/s molecules can be attempted.

Acknowledgements

A. J. Day is supported by the Arthritis & Rheumatism Council (grant no D0067). P. N. Barlow, A. Steinkasserer and I. D. Campbell work within the Oxford Centre for Molecular Sciences (OCMS).

References

- Arlaud, G. J., Thielens, N. M. & Aude, C. A. (1989) Structure and function of C1r and C1s: current concepts. *Behring Inst. Mitt.* 84, 56–64.
- Barlow, P. N., Baron M., Norman, D., Day, A. J., Willis, A. C., Sim, R. B. & Campbell, I. D. (1991) The secondary structure of the complement control protein module by two-dimensional ¹H-NMR. *Biochemistry* 30, 997–1004.
- Barlow, P. N., Norman, D., Steinkasserer, A., Horne, T. J., Pearce, J., Driscoll, P. C., Sim, R. B. & Campbell, I. D. (1992) Solution structure of the fifth repeat of Factor H: a second example of the complement control protein module. *Biochemistry* 31, 3626–3634.
- Barlow, P. N., Steinkasserer, A., Norman, D. G., Kieffer, B., Wiles, A. P., Sim, R. B. & Campbell, I. D. (1993) Solution structure of a pair of complement modules by NMR. *J. Mol. Biol.* 232, 268–284.
- Bisgrove, B. W., Andrews, M. E. & Raff, R. A. (1991) Fibroblasts, products of an EGF repeat-containing gene, from a unique extracellular matrix structure that surrounds the sea urchin embryo. *Dev. Biol.* 146, 89–99.
- Cohen, G. H., Silverton, E. W. & Davies, D. R. (1981) Refined crystal structure of g-chymotrypsin at 1.9 Å resolution. Comparison with other pancreatic serine proteases. *J. Mol. Biol.* 148, 449–479.
- Cooke, R. M., Wilkinson, A. J., Baron, M., Pastore, A., Tappin, M. J., Campbell, I. D., Gregory, H. & Sheard, B. (1987) The solution structure of epidermal growth factor. *Nature* 327, 339–341.
- Fothergill, J., Kemp, G., Paton, N., Carter, P. & Gray, P. (1989) The structures of human C1r and C1s and their relationship to other serine proteases. *Behring Inst. Mitt.* 84, 72–79.
- Handford, P. A., Mayhew, M., Baron, M., Winship, P. R., Campbell, I. D. & Brownlee, G. G. (1991) Key residues involved in calcium-binding motifs in EGF-like domains. *Nature* 351, 164–167.
- Hecht, P. M. & Anderson, K. V. (1992) Extracellular proteases and embryonic pattern formation. *Trends Cell Biol.* 2, 197–202.
- Hess, D., Schaller, J. & Rickli, E. E. (1991) Identification of the disulphide bonds of human C1r and C1s. *Biochemistry* 30, 2827–2833.
- Journet, A. & Tosi, M. (1986) Cloning and sequencing of full-length cDNA encoding the precursor of human complement C1r. *Biochem. J.* 240, 783–787.
- Leytus, S. P., Kurachi, K., Sakariassen, K. S. & Davie, E. W. (1986) Nucleotide sequence of cDNA coding for human complement C1r. *Biochemistry* 25, 4855–4863.
- Mackinnon, C. M., Carter, P. E., Smyth, S. J., Dunbar, B. & Fothergill, J. E. (1987) Molecular cloning of cDNA for human complement component C1s. *Eur. J. Biochem.* 169, 547–553.
- Matsumoto, M., Nagaki, K., Kitamura, H., Kuramitsu, S., Nagasawa, S. & Seya, T. (1989) Probing the C4-binding site on C1s with monoclonal antibodies. Evidence for a C4/C4b-binding site on the gamma-domain. *J. Immunol.* 142, 2743–50.
- Medved, L. V., Busby, T. F. & Ingham, K. C. (1989) Calorimetric investigation of the domain structure of human

complement C1s – reversible unfolding of the short consensus repeat units. *Biochemistry* 28, 5408–5414.

Norman, D. G., Barlow, P. N., Baron, M., Day, A. J., Sim, R. B. & Campbell, I. D. (1991) The three-dimensional structure of a complement control protein module in solution. *J. Mol. Biol.* 219, 717–725.

Perkins, S. J. (1989) Models for the C1 complex determined by physical techniques. *Behring Inst. Mitt.* 84, 129–141.

Perkins, S. J. & Nealis, A. S. (1989) The quaternary structure in solution of human complement C1r₂C1s₂. *Biochem. J.* 263, 463–469.

Reid, K. B. M. & Day, A. J. (1989) Structure-function relationships of the complement components. *Immunol. Today* 10, 177–181.

Sakiyama, H., Yamaguchi, K., Chiba, K., Nagata, K., Taniyama, C., Matsumoto, M., Suzuki, G., Tanaka, K., Tomoasawa, T., Yasukawa, M., Kuriwa, K. & Sakiyama, S. (1991) Biochemical characterisation and tissue distribution of hamster complement C1s. *J. Immunol.* 146, 183–187.

Sim, R. B. & Perkins, S. J. (1989) Molecular modelling of C3 and its ligands. *Curr. Top. Microbiol. Immunol.* 153, 209–222.

Steinkasserer, A., Barlow, P. N., Willis, A. C., Kertesz, Z., Campbell, I. D., Sim, R. B. & Norman, D. G. (1992) Activity, disulphide mapping and structural modelling of the fifth domain of human β_2 -glycoprotein I. *FEBS Lett.* 313, 193–197.

Thielens, N. M., Aude, C. A., Lacroix, M. B., Gagnon, J. & Arlaud, G. J. (1990a) Ca²⁺ binding properties and Ca²⁺-dependent interactions of the isolated N-terminal alpha fragments of human complement proteases C1r and C1s. *J. Biol. Chem.* 265, 14469–14475.

Thielens, N. M., van Dorsselaer, A., Gagnon, J. & Arlaud, G. J. (1990b) Chemical and functional characterisation of a fragment of C1s containing the epidermal growth factor homology region. *Biochemistry* 29, 3570–3578.

Yamaguchi, K., Sakiyama, H., Moriya, H., Matsumoto, M. & Sakiyama, S. (1990) Degradation of types I and II collagen by human C1s. *FEBS Lett.* 268, 206–211.

Zaccari, G., Aude, C. A., Thielens, N. M. & Arlaud, G. J. (1990) Neutron-scattering study of the γ B catalytic domains of complement proteases C1r and C1s. *FEBS Lett.* 269, 19–22.

Serpin Structures

R. A. ENGH, A. J. SCHUBERT

Max-Planck-Institut für Biochemie

Introduction

The strong interest in the serpin family is generated by its diverse biological roles, and the variety of its properties. Several dozen serpins have been identified among plants and higher animals; they are protease inhibitors, although some are identified with other functions. The serpins are the major protein make-up about 10% of the total protein content in plasma. They play roles in a diverse range of biological processes, including zymogen activation, blood coagulation, digestive processes, tumour invasion during metastasis, and genetic deficiencies have been identified of serpins. Many serpins show unusual mobile behaviour and rearrangements accompany their functions. This unusual behaviour with the first X-ray structure of the serpin α_1 -protein has been highlighted by the structure of the serpin on serpins. These features have been studied in an unprecedented way to study the adaptability of protein structure for the unfolding function, folding, and

Scope of this Paper

A large number of serpin structures have been determined over the past decade, describing the mechanism of inhibition (Engh, 1983), physiological importance (Patston, 1991), their role in disease (Crystal, 1991), to name a few. The discussion of spatial structure and function (Engh, 1989) has relied

STIC-ILL

From: Romeo, David
Sent: Monday, June 04, 2001 1:30 PM
To: STIC-ILL
Subject: 09503421 Rosano C L; Braun C B; Hechemy K E; Parhami N; Satoh P S; Hurwitz C

Borrower's Name ... David Romeo
Org or A.U. ... 1647, Mailbox, 10E18
Phone ... 305-4050
Serial Number ... 09503421
Date of Request ... 04 June 01
Date Needed by ... 06 June 01

PLEASE COPY REFERENCE

AU Rosano C L; Braun C B; Hechemy K E; Parhami N; Satoh P S; Hurwitz C
TI Inhibition of Clq functions by RHP, a protein elevated
in sera from patients with rheumatoid arthritis.
SO COMPLEMENT, (1988) 5 (2) 57-64.
Journal code: DOB; 8409977. ISSN: 0253-5076.
AN 88242190

349185

3267203

XO
6/4

Inhibition of C1q Functions by RHP, a Protein Elevated in Sera from Patients with Rheumatoid Arthritis

Carmen L. Rosano, Catherine B. Braun, Karim E. Hechemy, Nourollah Parhami, Paul S. Satoh, Charles Hurwitz

Research Service Veterans Administration Medical Center, and Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, N.Y.;
The Upjohn Co., Kalamazoo, Mich., USA

Key Words. C1q · RHP · Rheumatoid arthritis · Oxidative metabolism of polymorphonuclear neutrophils · Disaggregation of C1

Abstract. We have previously shown that serum levels of C1q, unbound to C1r × C1s, are elevated in rheumatoid arthritis. We have also shown that RHP, a newly described serum protein which affects the C1q-anti C1q precipitin reaction, is also present at elevated levels in rheumatoid arthritis. We now show that RHP inhibits the hemolytic activity of C1q, disaggregates C1, and inhibits the ability of C1q bound to latex beads or to aggregated IgG to enhance the oxidative metabolism of neutrophils.

Introduction

We have previously shown that serum levels of C1q unbound to C1r × C1s are elevated in patients with active rheumatoid arthritis (RA) uncomplicated by vasculitis [1], but not in patients with other arthritides [2, 3]. The finding of elevated serum levels of C1q in RA has been confirmed by Ochi et al. [4]. The significance of elevated C1q in RA is not clear. However, it has been shown that a variety of cells including neutrophils, macrophages and lymphocytes possess specific membrane receptors for C1q [5-9]. In addition,

Tenner and Cooper [10] have demonstrated that C1q bound to latex beads or to aggregated IgG, enhances the generation of oxygen radicals by neutrophils. Thus, C1q may play an important role in the pathogenesis of tissue damage in RA.

Recently, we have isolated and purified from the sera of patients with active RA a protein (designated RHP) which enhances the size of the C1q-anti C1q precipitin ring [11]. This protein has a molecular weight of 135 kilodaltons and an isoelectric point of 5.1-5.3. Immunological evidence suggests that RHP may be a newly discovered serum

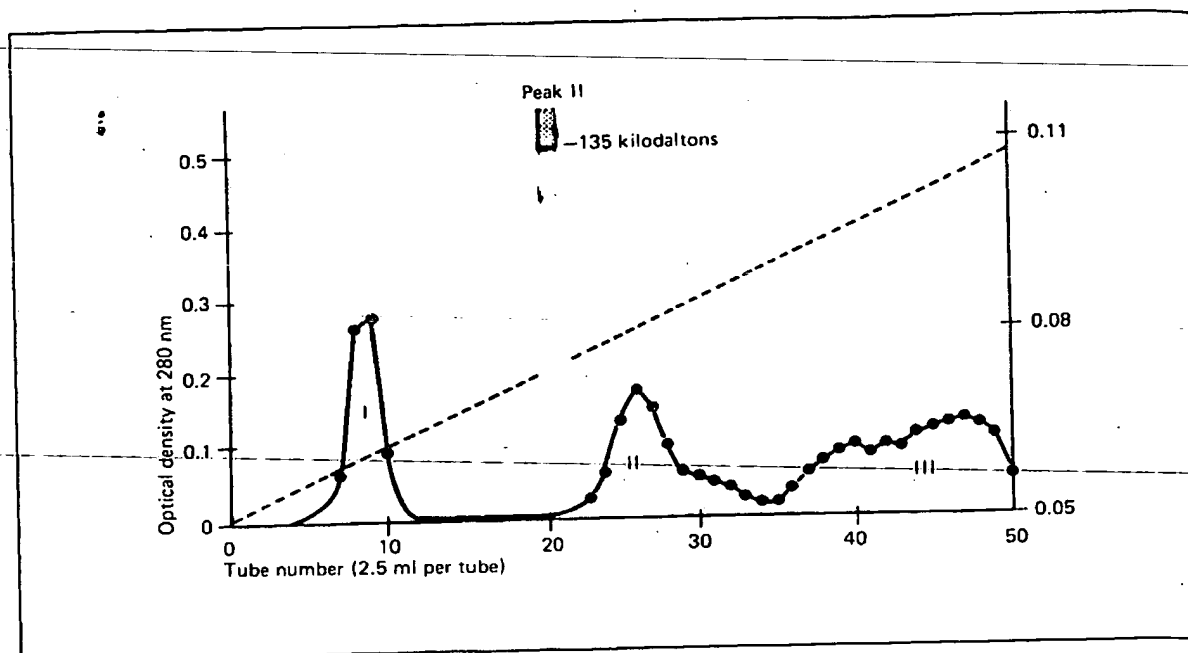


Fig. 1. Partial purification of RHP from the S1 extract of the euglobulin fraction by chromatography on DEAE-cellulose. The S1 fraction is a 0.026 M EGTA extract of the euglobulin fraction. The column was eluted with 0.05–0.15 M NaCl gradient in equilibrating buffer, pH 7.3. The details are described in Materials and Methods. The insert illustrates the SDS-PAGE of the proteins in peak II in the presence of β -mercaptoethanol.

protein since only one precipitin arc was formed by immunoelectrophoresis of RHP against anti whole human serum, and a single arc having the same electrophoretic mobility was formed when whole human serum was immunoelectrophoresed against anti-RHP. RHP also did not react with antisera against rheumatoid factor, C-reactive protein, orosomucoid, haptoglobin, C3, transferrin, hemopexin, alpha-1-antitrypsin, factor H, C4-binding protein, human glycoprotein, alpha-2-macroglobulin and ceruloplasmin. The possibilities that RHP might be either the chondroitin-4-sulfate C1q inhibitor or the C1q inhibitor isolated from lymphocyte membrane were also eliminated [12]. In the current study, we have demon-

strated that RHP disaggregated C1 complex, inhibits hemolytic activity of the complement system, and the ability of C1q to enhance the oxidative metabolism of neutrophils.

Materials and Methods

Isolation of RHP

RHP was isolated and purified by a procedure modified from that previously described [11]. The euglobulin fraction from 8 ml of pooled serum obtained from patients with active adult RA was dissolved in 4 ml of 0.5 M NaCl–0.01 M EDTA, and dialyzed for 18 h against 1,400 ml of 0.026 M EGTA, pH 7.5, to obtain the supernatant fraction (S1) which contained about 12 mg of protein. The S1 eluate was dialyzed against 0.04 M Tris buffer, pH 7.3, contain-

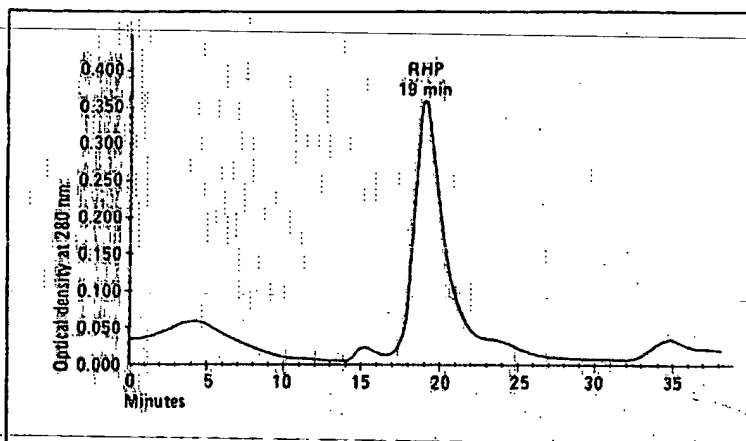
Fig. 2. Chromatogram of figure 1 in distilled water against sodium pH 6.8). The dialyzed fluid was applied to a column (10 cm) and eluted with 250 mM NaCl at 1 ml/min.

ing 0.002 M glucose (40 mg/ml) and equilibrated then eluted with 0.05 M NaCl. The purified water buffer (0.05 M phosphate, pH 7.3) was lyophilized and then high pressure column (10 cm) and eluted with 0.05 M NaCl and a flow rate of 1 ml/min.

Measurement of C1q

C1q was depleted from sheep red blood cells (SRBC) by incubation with 7.3, containing 0.05 M NaCl. Hemolysis was measured at 412 nm. That the mixture of indicated depleted SRBC 30 min red blood cells. That the C1q-depleted SRBC that hemolysis addition of

Fig. 2. High pressure liquid chromatography of the proteins in peak II of figure 1. The proteins in peak II of figure 1 were lyophilized, dissolved in distilled water, and dialyzed against equilibrating buffer (0.1 M sodium sulfate-sodium phosphate, pH 6.8). Two hundred microliters of the dialyzed proteins (80 μ g) were applied to a 60-cm high pressure liquid chromatography gel filtration column (Biorad TSK 250) which was eluted with the equilibrating buffer at 250 psi and a flow rate of 1 ml/min.



ing 0.002 M EDTA, and then applied to a DEAE-cellulose (40 \times 0.9 cm DE 52, Whatman, Clifton, N.J.) equilibrated with the same buffer. The column was then eluted with a 0.05–0.15 M NaCl gradient in equilibrating buffer as illustrated in figure 1.

The proteins in peak II were dissolved in 1 ml distilled water and then dialyzed against an equilibrating buffer (0.1 M sodium sulfate–0.02 M sodium phosphate, pH 6.8). Two hundred microliters of the dialyzed proteins from peak II were then applied to a high pressure liquid chromatography gel filtration column (Biorad TSK 250, 60 cm) which was isocratically eluted with the equilibrating buffer at 250 psi and a flow rate of 1 ml/min (fig. 2).

Measurement of Hemolytic Activity Initiated by C1q

C1q was added to 30 μ l of a 1:1 dilution of C1q-depleted serum [13] and 0.2 ml of a 1:5 dilution of sheep red blood cells (3×10^7) in Veronal buffer, pH 7.3, containing 0.1 % of bovine serum albumin. Hemolysis was measured as the increase in absorption at 412 nm after 25 min at 37 °C.

That the sheep red blood cells were sensitized by a mixture of IgG and IgM heterophile antibodies was indicated by the finding that exposure of the C1q-depleted serum to 10 mM β -mercaptoethanol for 30 min reduced the hemolytic activity by about 12%. That the complement system was otherwise intact in the C1q-depleted serum was shown by the finding that hemolysis of the sheep red blood cells required addition of C1q in a dose-dependent manner.

Determination of C1 and Its Subunits by Immunoelectrophoresis

Dissociation of C1 by RHP was measured by a modification of the procedure of Laurell et al. [14]. Thirty micrograms of C1r and of C1s (obtained from Dr. D.H. Bing, Center for Blood Research, Boston, Mass.) were added to 66 μ g of nonaggregated C1q (Calbiochem) and the mixture was dialyzed for 5 h at 4 °C against Tris-barbiturate buffer containing 2.5 mM Ca^{++} . To obtain nonaggregated C1q, the C1q obtained from Calbiochem was centrifuged for 10 min at 10,000 rpm. Twenty microliters of this dialyzed mixture were placed in each well in 1 % agarose containing the same buffer, and the equilibrium mixture was electrophoresed for 3 h at 100 V. The paths of migration of the gel strips were excised and again immunoelectrophoresed for 3 h at 100 V in 1 % agarose containing EDTA-Tris barbiturate buffer and a 1:40 dilution of anti-C1s (Atlantic Antibodies). After rinsing with saline and water followed by deproteinization, the precipitin areas corresponding to C1, C1r \times C1s and C1s were visualized by staining with Coomassie blue.

Measurement of Oxidative Metabolism of Neutrophils

Isolation of Human Neutrophils. A cell population consisting of over 95% neutrophils was obtained by mixing 30 ml of heparinized human blood with an equal volume of dextran solution (2% in saline) and allowing the red cells to sediment for 30 min. The supernatant fluid was removed by centrifugation at

1,000 g at 4°C for 20 min and the red cells in the pellet were lysed by suspension in a solution containing 9 parts of 0.83% ammonium chloride and 1 part 0.1 M Tris, pH 7.4. Lysis was allowed to continue for 5 min at 37°C. The residual cells were centrifuged at 1,000 g at 4°C for 20 min and resuspended in 5 ml of Hanks' balanced salt solution (HBSS). The cellular suspension was layered over 5 ml of Ficoll-Hypaque, centrifuged at 1,000 g at room temperature for 30 min, and the resulting neutrophil suspension was washed with 10 ml of HBSS.

Binding of C1q to Latex Beads. Polystyrene latex beads (5×10^5 , $15 \pm 8 \mu\text{m}$ in diameter, polystyrene beads, Sigma Chemical Co.) were washed 3 times with HBSS containing 0.02 M Hepes, pH 7.2, and incubated with 2,000 μg of purified nonaggregated C1q (Calbiochem) for 30 min at room temperature in 500 μl of the buffer. After 3 washings with the buffer, 4.8 μg C1q were found to be bound per 10^6 beads.

Binding of C1q to Aggregated IgG. Human IgG (10 mg/ml, Sigma Chemical Co.) was aggregated by incubation at 63°C for 20 min, and particulate aggregates were removed by centrifugation for 5 min at 10,000 g. A mixture of 300 μg of aggregated IgG and 30 μg of C1q was then incubated for 15 min at 22°C before adding the neutrophil suspension.

Measurement of Oxidative Metabolism of Neutrophils by Chemiluminescence

Oxidative metabolism of the neutrophils was monitored by the luminol-enhanced chemiluminescent response, according to the procedure of Tenner and Cooper [10]. Neutrophils (2.5×10^5 cells) were incubated in a final volume of 0.5 ml of HBSS containing 0.02 M Hepes at pH 7.2, 0.25% albumin and 2 μM luminol in 4 ml polypropylene tubes. Chemiluminescence was monitored in a Packard Model 4530 liquid scintillation counter in the single-photon counting mode.

Results

Inhibition by RHP of the Hemolytic Activity of the Complement System Initiated by C1q

Our finding that RHP affects the C1q-anti C1q precipitin reaction [11], suggested that it might affect other properties of C1q.

We therefore examined the effect of RHP on the hemolytic activity of the complement system initiated by C1q as measured by the procedure of Kolb et al. [13]. As illustrated in figure 3, the hemolytic activity induced by 0.25 μg of C1q was completely inhibited by 1.6 μg of RHP in the presence of C1r and C1s, estimated to be in 4-fold excess over that required to form C1 from the 0.25 μg of C1q as follows: 0.25 μg C1q added to 30 μl of 1:1 dilution of C1q-depleted serum is equivalent to 16.6 $\mu\text{g}/\text{ml}$. Normal serum contains 30 $\mu\text{g}/\text{ml}$ of C1r and of C1s, and 70 $\mu\text{g}/\text{ml}$ of C1q at a molar ratio of 2:2:1. The ratio of 70/16.6 is 4.2, indicating a 4-fold excess of $\text{C1r}_2 \times \text{C1s}_2$ under the conditions indicated.

Disaggregation of C1 by RHP

The possibility that the inhibitory effect of RHP on the hemolytic activity of the complement system might result from its ability to disaggregate C1 was tested by measuring the effect of RHP on the disaggregation of C1 by the technique of Laurell et al. [14]. Twenty microliters of the equilibrium mixture containing C1 formed from incubation of equivalent concentrations of C1 subunits in 2.5 mM Ca^{++} were added to each of the two wells in agarose gel, and 1 μg of RHP was added to one of the two wells. After electrophoresis and immunoelectrophoresis against anti-C1s at 10°C, the areas of C1, $\text{C1r} \times \text{C1s}$ and C1s were visualized by the Coomassie-blue-stained precipitin formed from the interactions with anti-C1s (fig. 4). In the absence of RHP, a precipitin area corresponding to C1 and a lesser area corresponding to $\text{C1r} \times \text{C1s}$ were obtained. In the presence of 1 μg RHP the C1 peak was greatly reduced and a large C1s peak appeared, indicating that almost all of the C1

active
indica
0.25

had
stead
excep
by a
A
(1:40
upon
resis
the is
buffe
finda
the d
result
form
ods

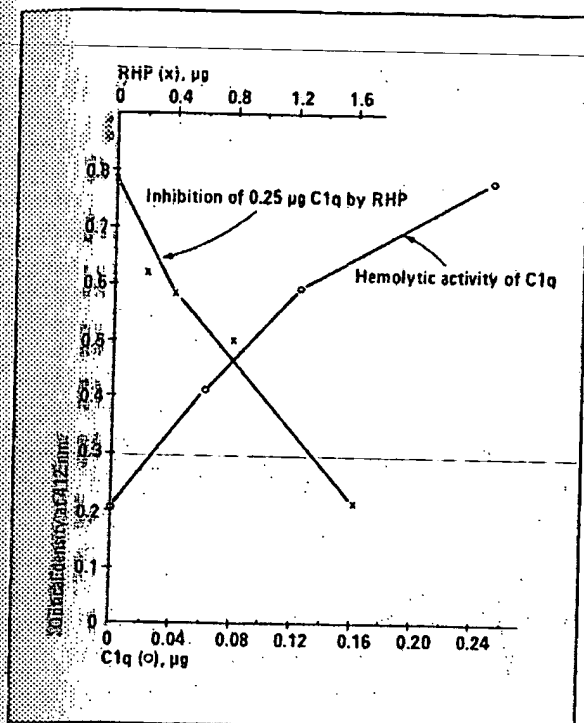
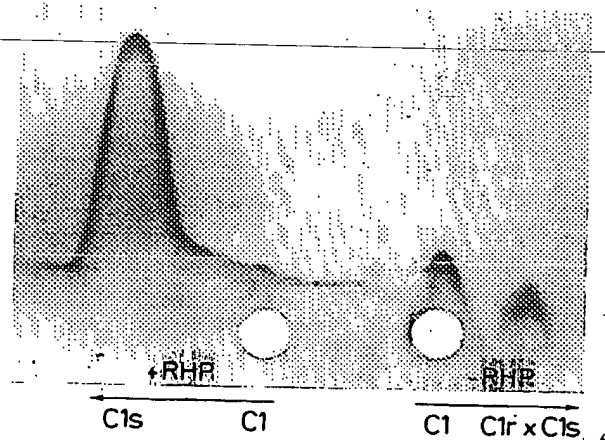


Fig. 3. Inhibitory effect of RHP on the hemolytic activity of the complement system initiated by C1q. Indicated amounts of RHP (0–1.6 μg) were added to 0.25 μg of C1q in 30 μl of 1:1 dilution of C1q-

had been disaggregated. Use of anti-C1r instead of anti-C1s produced similar results, except that the C1 peaks were not visualized by anti-C1r.

At the concentration of anti-C1s used (1:40 dilution) there was no effect of RHP upon electrophoresis or immunoelectrophoresis of C1s, or of C1r₂ × C1s₂ formed by the incubation of 30 μg of C1r and of C1s in buffer containing 2.5 mM Ca⁺⁺. The latter finding is of interest since it indicates that the disaggregation of C1 by RHP does not result from its sequestration of Ca⁺⁺. C1 formed as indicated in Materials and Methods and then disaggregated by addition of



depleted serum and 0.2 ml of a 1:5 dilution of activated sheep red blood cells in Veronal buffer, pH 7.3, containing 0.1% albumin.

Fig. 4. Disaggregation of C1 by RHP as measured by the Laurell et al. [14] technique. C1 prepared as described in 'Materials and Methods' was electrophoresed in the direction of the arrows and then immunoelectrophoresed against a 1:40 dilution of anti-C1s. The resulting precipitins were visualized by staining with Coomassie blue. The position of the peaks corresponding to C1, C1r × C1s and C1s are indicated on the figure.

EDTA presented a precipitin pattern similar to that observed in the presence of RHP in 2.5 mM Ca⁺⁺.

Measurement of Ca⁺⁺ Binding Capability of RHP

The possibility that RHP promoted the disaggregation of C1 by acting as a Ca⁺⁺ binder was investigated since aggregation of the subunits of C1 requires Ca⁺⁺. RHP, suspended in 0.026 M EGTA, pH 7.5, to remove bound Ca⁺⁺ if present, was then dialyzed free of EGTA, and exposed to 2.5 mM ⁴⁵Ca⁺⁺ (specific activity, 11 mCi/mg) in an equilibrium dialysis chamber at room tem-

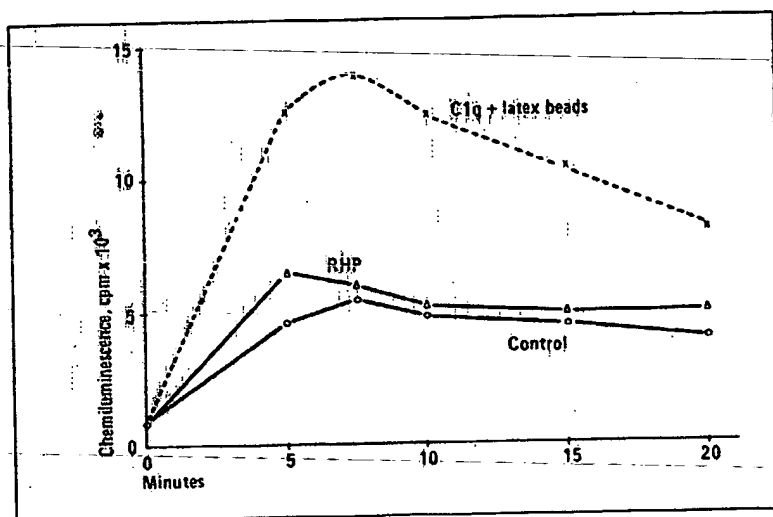


Fig. 5. Inhibition by RHP of the enhancement of the oxidative metabolism of neutrophils by C1q bound to latex beads. Oxidative metabolism of neutrophils (2×10^5 cells) incubated in HBSS containing 0.02 M Hepes, pH 7.2, and 0.25% albumin was monitored by luminol excitation as described in Materials and Methods. The control tube contained latex beads without C1q. The tube labeled C1q + latex beads contained 4.8 μ g C1q adsorbed to 1×10^6 latex beads, and the tube labeled RHP contained 16 μ g RHP in addition.

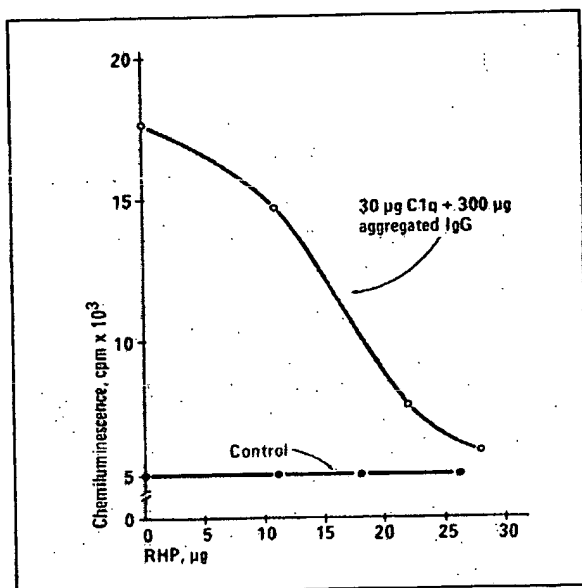


Fig. 6. Inhibition by RHP of the enhancement of the oxidative metabolism of neutrophils by C1q bound to aggregated IgG. The procedure was the same as described in figure 3, except that 30 μ g C1q adsorbed to 300 μ g IgG were added to 2×10^5 neutrophils instead of the C1q adsorbed to latex beads. The inhibitory effect of RHP is shown as the decrease in chemiluminescence at 10 min as a function of concentration of RHP. The control tube contained IgG without adsorbed C1q.

perature for 4 h. No increase in radioactivity in the chamber containing RHP was observed. The finding that RHP did not cause disaggregation of $C1r_2 \times C1s_2$ (as reported above) is further evidence that RHP does not promote disaggregation of C1 by acting as a Ca^{++} binder, since Ca^{++} is also required for the formation of this dimer.

Effect of RHP on the C1q Enhancement of the Oxidative Metabolism of Polymorphonuclear Neutrophils

Tenner and Cooper [10] have shown that C1q, but not C1, bound to latex beads or to aggregated IgG enhances the oxidative metabolism of neutrophils by an external excitation. The oxidative metabolism of neutrophils exposed to C1q bound to latex beads or to aggregated IgG was monitored by chemiluminescence. Figure 5 shows the enhancing effect of 4.8 μ g of C1q bound to 10^6 latex beads on luminol excitation by 2×10^5 neutrophils and the inhibition of this enhancement by RHP. The control curve shows the effects of latex beads alone

Inhibiti

while
ics of
to the
16 μ g
the en
As a c
10⁷ lat
were e
25°C
fluid w
and by
C1q w
4% of
exposu
Ten
that C
hances
phils.
almost
effect o

Disce

The
the new
is capa
the clas
tion and
C1 com
be resp
establis
complex
The po
binding
or that
the com
nated.

The
C1 com
ever, it

while the upper curve illustrates the kinetics of the enhancing effect of C1q adsorbed to the beads. The middle curve shows that 16 μ g of RHP almost completely eliminates the enhancing effect of the adsorbed C1q. As a control, 98 μ g of C1q bound to 2×10^7 latex beads suspended in 0.35 ml buffer were exposed to 75 μ g of RHP for 1 h at 25 °C. C1q released into the supernatant fluid was assayed for by its protein content and by radial immunodiffusion assay. No C1q was detected, indicating that less than 4% of the adsorbed C1q were released by exposure to RHP.

Tenner and Cooper [10] have also shown that C1q adsorbed to aggregated IgG enhances the oxidative metabolism of neutrophils. As shown in figure 6, 28 μ g of RHP almost completely inhibited the enhancing effect of 30 μ g of adsorbed C1q.

Discussion

The results presented demonstrate that the newly described serum protein RHP [11] is capable of inhibiting hemolytic activity by the classical pathway of complement activation and is also capable of disaggregating the C1 complex. Although the latter effect may be responsible for the former, it is not yet established that RHP disaggregates the C1 complex bound to erythrocyte membranes. The possibilities that RHP may prevent binding of $C1r_2 \times C1s_2$ to the Ig-bound C1q, or that RHP may affect other components of the complement system have not been eliminated.

The mechanism for the disaggregation of C1 complex by RHP is also not clear; however, it is not due to the chelation of Ca^{++} .

Our observation that RHP inhibits the ability of latex particles or aggregated IgG coated with C1q to stimulate oxidative metabolism of neutrophils is of interest since the serum levels of RHP [12] and of C1q, unbound to $C1r_2 \times C1s_2$ are elevated in patients with active RA uncomplicated by vasculitis [1].

The inhibition of the classical pathway of complement activation by RHP (fig. 3), presumably by disaggregating C1 (fig. 4), suggests that RHP may, like C4-binding protein [15], be a regulator of the classical pathway of C3 activation. Like RHP, C4bp has been reported to be increased in RA [16].

Niven and Whaley [17] have described a factor in RA sera which inhibits the binding of C1q to immune complex and which reduces the capacity of serum to consume C4. It would be of interest to determine whether their serum factor is RHP, since RHP affects a number of properties of C1q, as described in the text, and the disaggregation of C1 by RHP (fig. 4) should lead to reduced activation of C4.

The presence of specific receptor sites for dissociated C1q on numerous cell types, and the recent report that B cell receptor-bound C1q inhibits constitutively produced 'IL 1-like' activity [18] indicates that the elevated serum levels of dissociated C1q and of RHP in RA may play important roles in the immunoregulation of this autoimmune disease.

Acknowledgement

This work was supported in part by the Research Service of the Veterans Administration. We wish to acknowledge the excellent technical assistance of John A. Fox, and the valuable critiques of Dr. Min-Fu Tsan.

References

- 1 Rosano, C.L.; Parhami, N.; Hurwitz, C.: Serum levels of C1 subunits in rheumatoid arthritis. *J. Lab. clin. Med.* 103: 313-321 (1984).
- 2 Rosano, C.L.; Parhami, N.; Hurwitz, C.: The clinical significance of protein-bound hydroxyproline fractions in sera of patients with adult rheumatoid arthritis. *J. Rheum.* 6: 656-663 (1979).
- 3 Rosano, C.L.; Parhami, N.; Hurwitz, C.: Determination of C1q in human sera. *J. Lab. clin. Med.* 94: 593-599 (1979).
- 4 Ochi, T.; Yonemasu, K.; Ono, K.: Immunochemical quantitation of complement components of C1q and C3 in sera and synovial fluids of patients with bone and joint diseases. *Ann. rheum. Dis.* 39: 235-240 (1980).
- 5 Dickler, H.B.; Kunkel, H.G.: Interaction of aggregated gamma-globulin with B lymphocytes. *J. exp. Med.* 136: 191-196 (1972).
- 6 Gabay, Y.; Perlman, H.; Perlman, P.; Sobel, A.T.: A rosette assay for the determination of C1q receptor-bearing cells. *Eur. J. Immunol.* 9: 797-801 (1979).
- 7 Tenner, A.J.; Cooper, N.R.: Analysis of receptor-mediated C1q binding to human peripheral blood mononuclear cells. *J. Immunol.* 125: 1658-1664 (1980).
- 8 Tenner, A.J.; Cooper, N.R.: Identification of types of cells in human peripheral blood that bind C1q. *J. Immunol.* 126: 1174-1179 (1981).
- 9 Veerhuis, R.; Klar-Mohamad, N.; van Es, L.A.; Daha, M.R.: Enhanced binding and degradation of the C1q subcomponent of complement by thiolglycollate-stimulated guinea pig peritoneal macrophages. *Scand. J. Immunol.* 23: 581-587 (1986).
- 10 Tenner, A.J.; Cooper, N.R.: Stimulation of a polymorphonuclear leukocyte oxidative response by the C1q subunit of the first complement component. *J. Immunol.* 128: 2547-2552 (1982).
- 11 Rosano, C.L.; Parhami, N.; Hurwitz, C.: Isolation and purification of a C1q-anti C1q precipitin ring enhancing protein from sera of patients with rheumatoid arthritis. *Biochem. biophys. Res. Commun.* 128: 1288-1294 (1985).
- 12 Hurwitz, C.; Rosano, C.L.; Hechemy, K.E.; Satoh, P.S.: Biochemical and immunological properties of RHP, a serum protein associated with rheumatoid arthritis (Abstract). *Fed. Proc.* 46: 612 (1987).
- 13 Kolb, W.P.; Kolb, L.M.; Podack E.R.: C1q: isolation from human serum in high yield by affinity chromatography and development of a highly sensitive hemolytic assay. *J. Immunol.* 122: 2103-2111 (1979).
- 14 Laurell, A.B.; Martensson, U.; Sjöholm, A.G.: C1 subcomponent complexes in normal and pathological sera studied by crossed immunoelectrophoresis. *Acta pathol. microbiol. scand., C, Immunol.* 84: 455-464 (1976).
- 15 Fujita, T.; Nussenzweig, V.: The role of C4-binding protein and beta 1H in proteolysis of C4b and C3b. *J. exp. Med.* 150: 267-276 (1979).
- 16 Delamere, J.P.; Scott, D.L.; Bradwell, A.R.; King, T.C.: Plasma levels of C4 binding protein and beta 1H in rheumatoid arthritis. *Ann. rheum. Dis.* 40: 190-191 (1981).
- 17 Niven, I.P.; Whaley, K.: Inhibition of C1q binding to antigen-antibody complexes by a factor in rheumatoid arthritis. *Rheumatol. int.* 6: 205-208 (1986).
- 18 Habicht, G.S.; Beck, G.; Ghebrehiwet, B.: C1q inhibits the expression of B lymphoblast cell line interleukin 1 (IL 1). *J. Immunol.* 138: 2593-2597 (1987).

Received: April 2, 1987

Accepted after revision: November 17, 1987

Dr. Charles Hurwitz
Chief, Basic Science Research
Research Service
Veterans Administration
Medical Service 151B
113 Holland Avenue
Albany, NY 12208 (USA)

Co

O
RJ.F.
R.F.How
Div
WasKey
Rg bloAbs
discuss
(E) W
C4b* G
to E. F
fragme
system
C4 is
normal

Intro

The
blood g
turally
compos
ref. 1]
within
comple
two du
termed
related

Complement-dependent Proinflammatory Properties of the Alzheimer's Disease β -Peptide

By Bonnie M. Bradt,* William P. Kolb,[†] and Neil R. Cooper*

From the *Department of Immunology, The Scripps Research Institute, La Jolla, California 92037; and [†]Advanced Research Technologies, Inc., San Diego, California 92121

Summary

Large numbers of neuritic plaques (NP), largely composed of a fibrillar insoluble form of the β -amyloid peptide ($A\beta$), are found in the hippocampus and neocortex of Alzheimer's disease (AD) patients in association with damaged neuronal processes, increased numbers of activated astrocytes and microglia, and several proteins including the components of the proinflammatory complement system. These studies address the hypothesis that the activated complement system mediates the cellular changes that surround fibrillar $A\beta$ deposits in NP. We report that $A\beta$ peptides directly and independently activate the alternative complement pathway as well as the classical complement pathway; trigger the formation of covalent, ester-linked complexes of $A\beta$ with activation products of the third complement component (C3); generate the cytokine-like C5a complement-activation fragment; and mediate formation of the proinflammatory C5b-9 membrane attack complex, in functionally active form able to insert into and permeabilize the membrane of neuronal precursor cells. These findings provide inflammation-based mechanisms to account for the presence of complement components in NP in association with damaged neurons and increased numbers of activated glial cells, and they have potential implications for the therapy of AD.

Key words: Alzheimer's disease • amyloid • C3 • complement • inflammation

We and others (1–3) have noted that the pathological changes which characterize Alzheimer's disease (AD)¹ could all result from complement activation in neuritic plaques (NP), since this effector system has the ability to activate various cell types with release of cytokines and secondary mediators; to induce directed migration of these cells toward the complement activator; to alter cellular functions; and to damage cells (4, 5). Potential complement involvement in the brain is not dependent on disruption of the blood–brain barrier, since neurons, astrocytes, microglia, and oligodendrocytes synthesize most, and likely all, of the proteins of the complement system (6).

Since activation is a prerequisite for manifestation of all of the biological activities of the complement system, the β -amyloid peptide ($A\beta$) or another component of NP must possess the ability to activate complement in order for complement to be involved in mediating the pathologic cellular characteristics of AD. In this regard, we and others

previously showed that fibrillar forms of $A\beta$ bound the first reacting factor of the classical complement pathway (CCP), C1q (3, 7), and depleted the activity of the fourth complement component (C4) as well as whole complement activity (CH50), when incubated with human serum as a complement source in vitro (3, 7). Residues 14–26 of the collagen-like portion of the A polypeptide chain of the C1q molecule were implicated in binding fibrillar $A\beta$ (7). Recent studies have confirmed this suggestive evidence of CCP activation by aggregated $A\beta$ (8–12), and have also emphasized the critical role of the β -pleated structure of $A\beta$ in mediating these effects (9, 11). Inhibition studies have implicated $A\beta$ residues 1–11 in C1q binding (11, 12). The complement depletion, inhibition, and cleavage assays used in these various studies have provided suggestive evidence for CCP activation by fibrillar $A\beta$; however, as indirect assays, they are subject to other interpretations. In this context, we recently presented preliminary evidence suggesting that $A\beta$ forms complexes with C3 after incubation of fibrillar $A\beta$ with a complement source (9).

In the course of these studies, we found that the addition of fibrillar $A\beta$ to a complement source led to the generation of covalent ester-linked complexes of $A\beta$ with C3 activation fragments, providing unequivocal evidence for complement activation by $A\beta$, since covalent attachment

¹Abbreviations used in this paper: ACP, alternative complement pathway; AD, Alzheimer's disease; $A\beta$, β -amyloid peptide; CCP, classical complement pathway; dd, double distilled; MAC, membrane attack complex; NP, neuritic plaques; NHS, normal human serum; SOD, superoxide dismutase.

of C3 activation fragments to complement activators represents a fundamental tenet of complement action. We also found that fibrillar A β possesses the ability to activate the alternative complement pathway (ACP) in serum, as well as in mixtures of the six purified proteins of the alternative pathway in physiologic concentrations, providing the first indication that A β independently activates both complement pathways. Additionally, we observed that such activation is highly specific for A β and completely independent of oxidative processes. These studies are described here. Finally, we also report for the first time that A β -mediated complement activation is biologically significant, as it leads to generation of the cytokine-like C5a complement-activation fragment, and mediates formation of the proinflammatory C5b-9 membrane attack complex (MAC), in functionally active form able to insert into and permeabilize the membranes of neuronal precursor cells.

Materials and Methods

A β -Mediated Complement Activation and Complement Activation ELISA Assays. A β 1–40 and 1–42 (Bachem California, Torrance, CA; Bachem Bioscience, Inc., King of Prussia, PA; Quality Controlled Biochemicals, Inc., Hopkinton, MA; Anaspec, Inc., San Jose, CA; and California Peptide Research, Inc., Napa, CA) were dissolved in either 100% DMSO at 10 mg/ml, or double distilled (dd)H₂O, gradually diluted in ddH₂O to 2 mg/ml and then brought to 1 mg/ml in 0.1 M Tris buffer, pH 7.4. A β was used immediately (nonaggregated) or permitted to aggregate by incubation at room temperature for 48 h (A β 1–42) to 72 h (A β 1–40); further incubation for 2 wk did not decrease complement-activating potential. Preaggregated A β preparations were incubated with an equal volume of 1:5 normal human serum (NHS), complement-depleted sera (Advanced Research Technologies, Inc.), or the six purified proteins of the ACP (Advanced Research Technologies, Inc.) in physiologic ratios (13). Dilutions were in veronal buffered saline, pH 7.4, containing calcium and magnesium. Inhibition studies were carried out with preaggregated A β 1–42 in the presence of deferoxamine, glutathione, dimethylthiourea, catalase, or superoxide dismutase (SOD), all purchased from Sigma Chemical Co. (St. Louis, MO). After 1 h at 37°C, EDTA was added to stop further complement activation, and the samples were diluted (1:20–1:300) and added, in replicate, to microtitration wells precoated overnight (4°C) with 1 μ g (100 μ l) of mAb to A β (10D5), mAb to a C3b neoantigen (clone 129), or mAb to an iC3b neoantigen (Quidel, San Diego, CA) at pH 7.4, and then blocked (BLOTTO; Pierce Chemical Co., Rockford, IL). After 1 h at room temperature, bound A β –C3b/iC3b complexes were detected with rabbit Ab to C3 or to A β , horseradish peroxidase-conjugated anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and ABTS (Kirkegaard & Perry Laboratories). C3 standard curves were generated with dilutions of purified iC3b (Advanced Research Technologies, Inc.) captured on wells precoated with anti-iC3b (Quidel). C5b-9 was captured on wells precoated with mAb to a C5b-9 neoantigen located in the poly C9 portion of the complex (Quidel) and detected with polyclonal goat Ab to C6 followed by horseradish peroxidase-conjugated mouse anti-goat IgG (Accurate Chemical and Science Corp., Westbury, NY) and ABTS. Values for the C5b-9 assay were back-calculated to the values in undiluted NHS. In some experiments, neuropeptide Y-porcine, urotensin I, or exendin 3

(all from California Peptide Research, Inc.); insulin B chain (Sigma Chemical Co.); amyloid precursor protein-peptide (657–676 (Bachem California); and adenovirus penton base 50-residue fragment (residues 317–366; reference 14), as well as preaggregated A β 1–42 preparations and monomeric A β 1–42, were dissolved in DMSO or ddH₂O and aged at room temperature, exactly as described above for A β . In some studies, peptides, after dissolution as just described but diluted in Hepes-buffered NaCl at pH 7.4, were covalently cross-linked at a concentration of 200 μ M with the primary amine reactive agent, bis(sulfosuccinimidyl)suberate (BS³; Pierce Chemical Co.), at a concentration of 5 mM. After 30 min, the reaction was terminated by quenching. All peptides were incubated with NHS for 1 h at 37°C. After dilution, complement activation was assessed by the conventional CH50 assay (15) by quantitating residual functional C3 using C3-depleted serum according to product instructions (Advanced Research Technologies, Inc.), or by evaluating C5b-9 formation.

Studies with Hydroxylamine. After capture of complexes onto 10D5-coated wells, replicate wells were treated with 0.1 M Tris, pH 9.5, or 1 M hydroxylamine in 0.1 M Tris, pH 9.5, for 2 h at 37°C. After washing, remaining bound C3 was detected as described above. Residual A β was detected with rabbit Ab to A β , as described above. The formation of covalent complexes of A β with C3 activation products was also evaluated using the Western blotting procedure on SDS-PAGE gels. Replicate samples of aggregated A β 1–42 or A β 1–40 were incubated with human serum for 1 h at 37°C in the presence or absence of EDTA, and the reaction mixtures were then microfuged, washed in Tris buffer, and incubated for 3 h at 37°C with 0.1 M Tris at pH 7.4, 0.1 M Tris at pH 9.5, or 1 M hydroxylamine in 0.1 M Tris at pH 9.5. The samples were again microfuged, and washed with Tris at pH 7.4 followed by the same buffer containing 0.1% SDS. The samples were then subjected to SDS-PAGE under nonreducing conditions and electroblotted, and bands were detected with rabbit Ab to C3 followed by goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) and the SuperSignal system (Pierce Chemical Co.). After stripping, A β was detected with 6E10 mouse Ab to A β (Senetek PLC, St. Louis, MO) followed by goat anti-mouse IgG (Kirkegaard & Perry Laboratories) and the SuperSignal system. In some experiments, electroblotted gels were reacted first with rabbit Ab to A β (generated in this laboratory) or with 6E10 mAb to A β (Senetek PLC) followed by goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) or goat anti-mouse IgG (Kirkegaard & Perry Laboratories), stripped, and reacted with mAb anti-iC3b (Quidel) or rabbit Ab to C3 (generated in this laboratory), followed by goat anti-mouse IgG or goat anti-rabbit IgG. Quantitation was with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Mass Spectroscopy. After solubilization in 70% formic acid, samples were analyzed by MALDI spectroscopy (Perceptive Voyager ELITE; Perceptive Biosystems, Inc., Framingham, MA).

C5a. C5a (and C5a des-Arg) was detected in diluted samples with the Biotrak radioimmunoassay kit (Amersham Corp., Arlington Heights, IL); the samples were subjected to acid precipitation before analysis (16). Values were back-calculated to the concentrations in undiluted NHS.

C5b-9 Membrane Insertion. Ntera2/D1 (NT2) cells (Stratagene Inc., La Jolla, CA) were grown to subconfluence, released with nonenzymatic cell dissociation solution (GIBCO BRL, Gaithersburg, MD), washed, and resuspended (2 \times 10⁷ cells/ml). NT2 cells (100 μ l) were incubated with 50 μ l NHS in the presence or absence of EDTA and 50 μ l preaggregated A β . After 15 min at 37°C, the MAC was detected with rabbit Ab (Advanced Re-

search Technologies, Inc.) or mAb (Quidel) to C5b-9 neoantigens, followed by FITC anti-rabbit or -mouse Ig and propidium iodide. Readings were performed on a FACScan® and analyzed with CellQuest software (Becton Dickinson, San Jose, CA).

Results and Discussion

Sandwich-type ELISAs showed that complexes containing A β and C3b/iC3b were generated in NHS, as a complement source, after incubation with aggregated A β 1-42. Complexes were demonstrable after capture with mAbs to activation-dependent neoantigens in the first (C3b) or second (iC3b) C3 cleavage products and detection with rabbit Ab to A β (Fig. 1, *a* and *b*), as well as after capture with mAb to A β and detection with rabbit Ab to C3 (Fig. 1 *d*) or C3d (not shown). EDTA, which blocks complement activation by chelating calcium and magnesium, prevented complex formation (Fig. 1 *d*). ELISAs in which complexes were captured with mAb to A β and detected with Ab to C3 were used for most of the studies, since such ELISAs permitted quantitation by reference to included standard curves generated with purified C3 captured on wells coated with mAb to C3 and detected with rabbit Ab to C3 (Fig. 1 *d*). Complement activation was detectable to $\sim 1 \mu\text{M}$ A β 1-42 (Fig. 1 *d*). 10 different preaggregated A β 1-40 and 20 different preaggregated A β 1-42 preparations from 5 manufacturers generated such complexes. A β 1-42 was generally 5-10-fold more active than A β 1-40 in this regard. The cation-dependent formation of A β -C3b/iC3b complexes after incubation of aggregated A β with NHS provides unequivocal evidence for complement activation by aggregated A β .

A β -C3b/iC3b complex formation was evident after incubation of aggregated A β 1-42 with NHS lacking factor B, an essential component of the ACP (Fig. 1 *e*); such sera contain an intact CCP, but do not permit ACP activation. A significant reduction in complex formation was also evident in C1q-depleted serum compared with NHS (Fig. 1 *e*). These data show that the CCP mediates complex formation by aggregated A β , findings that were anticipated from the results of the complement depletion assays described earlier. Unexpectedly, however, the ACP also mediated the formation of complexes, since they were also generated after the addition of aggregated A β 1-42 to NHS lacking factor C1q, and complex formation was reduced in factor B-depleted serum compared with NHS (Fig. 1 *e*), a result replicated in four additional experiments with different A β 1-42 preparations. The ability of A β 1-42 to activate the ACP was confirmed in three studies in which preaggregated A β 1-42 was incubated with a mixture of the six purified proteins of the ACP (factors B, D, H, and I, properdin, and C3) in physiological ratios (reference 13; Fig. 1 *f*). These data document the ability of aggregated A β not only to activate the CCP, but also to independently activate the ACP. This is the first indication that aggregated A β activates the ACP; it had been presumed that complement activation by A β was exclusively via the CCP because of the absence of ACP components (factor B and properdin) in NP (1, 17, 18). The failure to detect ACP components in NP may be due to the extreme lability of the ACP C3 convertase.

Multiple different aggregated A β 1-42 preparations activated complement, as determined by the classical CH50 complement consumption technique (Fig. 1 *g*). The aging

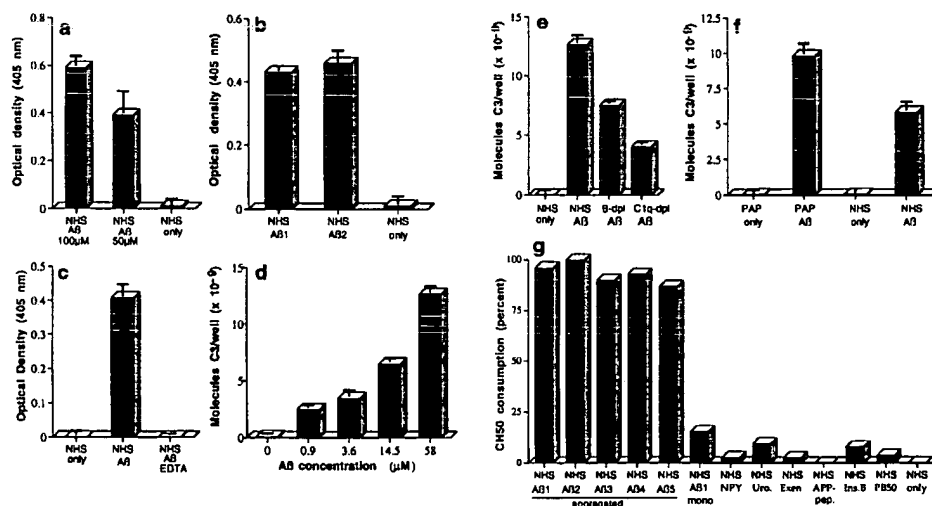


Figure 1. ELISA demonstration of complement-mediated formation of complexes of A β with C3 activation fragments (*a-d*). Complexes were captured, detected, and quantitated as described in Materials and Methods. NHS or purified ACP proteins only do not contain A β . (*a*) Preaggregated A β 1-42 (100 μM and 50 μM) was incubated in NHS, captured with mAb to C3b, and detected with rabbit Ab to A β . (*b*) Two preaggregated A β 1-42 preparations (A β 1 and A β 2, at 20 μM) were incubated in NHS, captured with mAb to iC3b, and detected with rabbit Ab to A β . (*c*) Preaggregated A β 1-42 (58 μM) was incubated in NHS, or in NHS containing 10 mM EDTA, captured with mAb to A β , and detected with rabbit Ab to C3. (*d*) Preaggregated A β 1-42 (58 μM) was incubated in NHS, factor B-depleted NHS (*B-dpl*), or C1q-depleted NHS (*C1q-dpl*), captured with mAb to A β , and detected with rabbit Ab to C3. (*e*) Preaggregated A β 1-42 (58 μM) was incubated with the six purified ACP proteins (*PAP*) or NHS, captured with mAb to A β , and detected with rabbit Ab to C3. Background levels obtained in EDTA controls containing A β and purified ACP proteins or NHS, in the various experiments described above, were subtracted. (*f*) Specificity of complement activation. Preaggregated A β preparations (20 μM) and the same concentrations of monomeric A β (*mono*), insulin B chain (*Ins.B*), neuropeptide Y-porcine (*NPY*), urotensin I (*Uro*), exendin 3 (*Exen*), amyloid precursor peptide 657-676 (*APP-pep*), and adenovirus penton base 50-residue peptide (*PB50*) were incubated with NHS. Complement activation was assessed by the CH50 method. Correlation coefficients for the CH50 determinations ranged from 0.995 to 1.000.

procedure used to aggregate A β generates β -pleated fibrils (9). Nonfibrillar "amorphous" aggregates of A β are devoid of complement-activating ability (9). In contrast to the aggregated preparations, A β used immediately after dissolution had limited ability to activate complement (Fig. 1 g). These data document the important role of fibril formation for complement activation by A β in vitro. Amylin, another peptide which spontaneously forms β -pleated fibrils, was also tested for complement-activating ability in these studies. The 37-residue amylin polypeptide represents the principal constituent of the amyloid deposits in type 2 diabetes. On SDS-PAGE gels, aged amylin migrated primarily as large SDS-insoluble stained bands. However, this fibrillar peptide did not significantly activate complement at a concentration of 100 μ M (7% CH50 consumption).

The specificity of complement activation by A β was also evaluated by determining whether complement was activated by other small peptides (20–50 amino acids) containing multiple residues able to mediate covalent linkage to the glutamate residue of the hydrolyzed thioester of C3 (serine, tyrosine, threonine, lysine) and expressing similar overall charge to A β . All of the peptides were processed and aged in the same manner as A β . None of the peptides, including the insulin B chain (30 residues), neuropeptide Y-porcine (36 residues), urotensin I (41 residues), exendin 3 (39 residues), amyloid precursor peptide 657–676 (20 residues), and the adenovirus penton base fragment (50 residues) significantly activated complement at a concentration of 20 μ M, as assessed by the classical CH50 technique (Fig. 1 g). The peptides also showed little or no ability to activate complement in other assays, including the ability to deplete residual functional C3 and form the SC5b-9 complex (not shown). To determine whether peptide aggregation would increase complement-activating ability, urotensin I, neuropeptide Y-porcine, and exendin 3 were covalently cross-linked with the primary amine reactive reagent BS³ before evaluating their ability to activate complement by the CH50 technique. Cross-linked urotensin I and neuropeptide Y-porcine gave a ladder of Coomassie-stained bands on SDS-PAGE analyses, but exendin 3 gave no stained bands, possibly due to the formation of very large aggregates. These three cross-linked peptides did not significantly activate complement (<10% CH50 depletion) at a concentration of 20 μ M. In another study, cross-linked urotensin I exhibited 7% CH50 consumption at a concentration of 100 μ M, whereas aggregated A β 1–42 showed 45% consumption. These data cumulatively demonstrate the marked specificity of complement activation by fibrillar A β .

C3 preferentially binds to activators via ester bonds, although amide linkage has been described (19, 20); such bonds form between the reactive γ -carbonyl group of the glutamate residue of the activation-cleaved internal thioester bond in C3, and hydroxyl (ester) or amino (amide) groups on the activator (19). To evaluate possible ester linkage, complexes were captured with mAb to A β and incubated with Tris buffer containing 1 M hydroxylamine at pH 9.5 for 2 h at 37°C, a treatment which disrupts ester but not amide bonds (19, 20). Approximately 50% of the bound

C3, but none of the A β , was removed from the captured complexes by this treatment (Fig. 2, a and b), a result replicated in two additional studies with A β 1–40 and 1–42.

The formation of covalent complexes of A β with C3 activation products was also independently demonstrated using a Western blotting approach. In these studies, aggregated A β 1–40 was incubated with serum, and the complexes of insoluble fibrillar A β with C3 activation fragments were then sedimented, washed, and incubated with either 1 M hydroxylamine at pH 9.5 or control buffers. After washing, a prominent band with a molecular mass of \sim 180 kD, the molecular mass of C3, as well as several higher molecular mass bands, were detected with Ab to C3 (Fig. 2 d). After stripping, the same bands were also found to react with Ab to A β , although the gels were darker due to the presence of large amounts of aggregated A β (Fig. 2 d). Bands of the same molecular masses reactive with Abs to both A β and C3 were also observed when the blotting studies were performed in the reverse direction, i.e., blotting first with either mAb or polyclonal Ab to A β followed, after stripping, by blotting with rabbit Ab or mAb to C3 (not shown). The 180-kD band and the larger bands, which contain both C3 and A β , undoubtedly represent complexes of A β monomers with C3b monomers and oligomers, since they were not evident in the reactions carried out in the presence of EDTA or in the absence of fibrillar A β (Fig. 2 d).

C3 was also detected in the large A β aggregates on the top of the gels (except for the EDTA lane) on longer exposure (not shown). The lesser reactivity of C3 in the larger A β aggregates at the top of the gels, compared with the C3 monomers and oligomers within the gels, indicates that not all A β monomers bear a molecule of C3b; this is not surprising, since A β is in large aggregates and, in addition, in molar excess over C3. It may also be that A β molecules bearing covalently bound C3b dissociate from the aggregates, in analogy to the dissociation of immune complexes by the covalent binding of C3b (21, 22).

Hydroxylamine treatment disrupted approximately half of the complexes (Fig. 2 d). Quantitative scanning of the C3 Western blot showed that the treatment with 1 M hydroxylamine at pH 9.5 removed 42% of the bound C3 compared with the pH 9.5 control; the pH 9.5 buffer treatment removed only trivial amounts (5.1%) of the bound C3 compared with the pH 7.4 treatment. The A β Western blot could not be satisfactorily scanned due to the large background, but visual inspection reveals the same pattern (Fig. 2 d). Identical results were obtained with A β 1–42 (not shown). These two independent assay systems both show that ester bonds, in part, mediate covalent attachment of C3 activation fragments to A β . A β 1–42 contains two serines, at positions 8 and 26, and a tyrosine, at position 10, which could mediate ester linkages with C3 activation fragments.

With regard to the bond(s) responsible for the nonester-linked A β -C3b/iC3b complexes, A β alone has been reported to generate free radicals (23) upon incubation in aqueous solution, and oxidative processes have been associated with A β denaturation, fragmentation, and oxidation (24, 25). Because of the potential relevance of these pro-

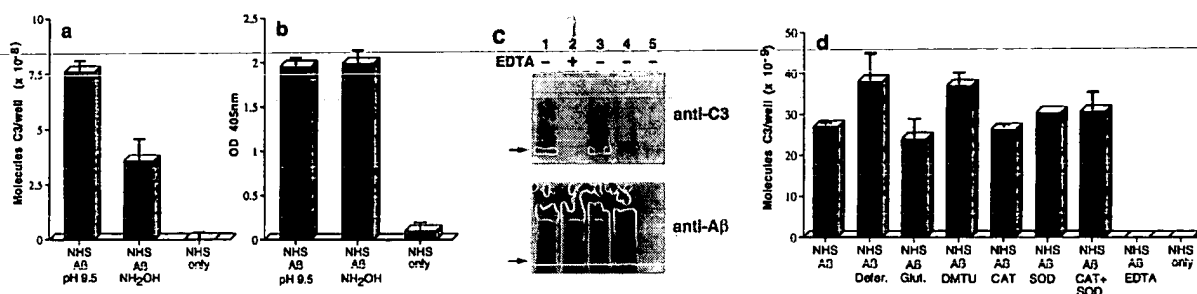


Figure 2. Assessment of bonds mediating binding of A β to C3 activation fragments. (a) Preaggregated A β 1–42 (25 μ M) was incubated in NHS, and complexes were captured on 10D5-coated wells; NHS only does not contain A β . Replicate samples were treated with pH 9.5 buffer, or with the same buffer containing 1 M hydroxylamine (NH₂OH), and remaining bound C3 was then detected and quantitated as described in Materials and Methods. The control containing A β , NHS, and EDTA has been subtracted. (b) Replicate wells subjected to treatment with the pH 9.5 buffer or hydroxylamine were evaluated for residual bound A β as described in Materials and Methods. (c) Preaggregated A β 1–40 was incubated in NHS in the presence or absence of EDTA; lane 5 contains NHS but no A β . After centrifugation and washing, fibrillar A β pellets were incubated with pH 7.4 buffer (lanes 1 and 2), pH 9.5 buffer (lane 3), or 1 M hydroxylamine in pH 9.5 buffer (lane 4). After further washing, samples were subjected to SDS-PAGE under nonreducing conditions followed by blotting for the presence of C3 and, after stripping, for A β . Arrow, The C3 band at \sim 180 kD. (d) Preaggregated A β 1–42 (50 μ M) was incubated in NHS alone, and in the presence of deferoxamine (Defer.; 1 mM), glutathione (Glut.; 1 mM), dimethylthiourea (DMTU; 30 mM), catalase (CAT; 2×10^4 U/ml), SOD (10 μ M), or catalase plus SOD, and the A β complexes with C3 activation fragments were then detected as described in Materials and Methods.

cesses to the formation of complexes of A β with C3 activation fragments, A β 1–42 was assessed by MALDI mass spectroscopy after aging from 0 to 10 d. Aggregates are not detected in these assays, since the samples are dissolved in 70% formic acid for mass spectroscopic analysis. The molecular mass of the major peak in the various samples ranged from 4510 to 4514, and no other peaks were present, ruling out significant oxidation, fragmentation, and covalent cross-linking of A β . To determine whether oxidative processes mediated the formation of complexes of A β with C3 activation fragments, complement activation was carried out in the presence of deferoxamine, glutathione, dimethylthiourea, catalase, SOD, and catalase plus SOD. Since none of these antioxidants or free radical scavengers inhibited the formation of or interfered with the detection of complexes (Fig. 2 d), it is unlikely that free radical-mediated or oxidative processes are involved in the formation of complexes of A β with C3 activation fragments. In all likelihood, amide bonds are responsible for the remaining A β –C3b/iC3b complexes. A β 1–42 contains two lysine residues, at positions 16 and 28, which could mediate such linkages.

Additional studies showed that A β triggered activation of the terminal, proinflammatory portion of the complement-reaction sequence in NHS. C5a, a cytokine-like activation cleavage product of C5 with numerous biological properties, was efficiently generated by aggregated A β 1–42 in NHS, as determined by a specific radioimmunoassay which detects C5a and C5a des-Arg (lacking the COOH-terminal arginine residue) (reference 26; Fig. 3 a). A sandwich ELISA in which an mAb to a C5b-9 neoantigen located in poly C9 served as the capture Ab, and polyclonal Ab to C6 served as the detection Ab, showed that A β -mediated complement activation led to formation of the C5b-9 complex (Fig. 3 b). This ELISA detects C5b-9 as well as SC5b-9 complexes; the latter are formed in NHS in the absence of

cells, as a consequence of the binding of S protein, a complement control protein, to the complex. A β 1–42 was generally more efficient in generating C5b-9 than A β 1–40 (Fig. 3 b). In contrast, another group recently reported that A β -mediated complement activation does not lead to generation of the C5b-9 complex (10). The reason(s) for their failure to demonstrate C5b-9 formation is not known. One possibility is the well-known variability in the properties of different A β preparations. In this regard, we have observed more variability in the ability of various A β 1–40 and A β 1–42 preparations to trigger C5b-9 complex formation, than in their ability to generate A β –C3b and A β –iC3b complexes. Other explanations could lie in slight differences in experimental conditions. For example, their C5b-9 formation experiments were carried out in NHS diluted 1:10 in phosphate-buffered NaCl; this combination provides suboptimal concentrations of calcium and magnesium, which are required for CCP and ACP activation. In this regard, we obtained 10-fold higher levels of SC5b-9 formation than they obtained with 1 μ M aggregated IgG in their control studies (not shown).

The C5b-9 complex generated by A β 1–42-mediated complement activation was able to insert into the membranes of NT2 cells, a committed neuronal precursor cell line, when such cells were included in reaction mixtures with aggregated A β and NHS (Fig. 4 a). Depicted are flow cytometric analyses with rabbit Ab to activation-specific neoantigens in the C5b-9 MAC. C5b-9 membrane insertion was likely proportional to the extent of complement activation, since it was dependent on the concentration of A β 1–42. mAb to C5b-9 neoantigens gave the same result (not shown). Identical A β 1–40 concentrations mediated lower levels of C5b-9 membrane insertion (not shown), probably because of the significantly lower levels of C5b-9 formation with A β 1–40. NT2 cells and other neuronal cell lines are resistant to complement-dependent cytotoxicity,

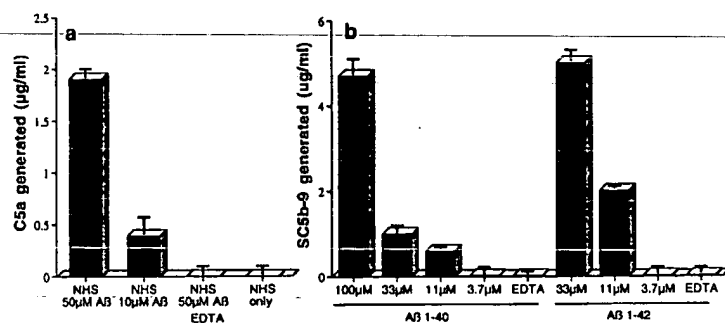


Figure 3. Aβ-mediated complement activation generates C5a and the MAC. (a) Preaggregated Aβ 1-42 was incubated in NHS, and C5a generation was then quantitated as described in Materials and Methods. (b) C5b-9 was quantitated after NHS was incubated with varying concentrations of preaggregated Aβ 1-42 or 1-40. SC5b-9 formation was quantitated as described in Materials and Methods.

likely because of the presence of CD59 (27), a complement regulatory protein, a finding confirmed here. Nevertheless, C5b-9 insertion into NT2 cell membranes mediated an increase in the permeability of the cells to propidium iodide that was dependent on the concentration of Aβ 1-42 (Fig. 4 b). These data indicate that C5b-9 generated by Aβ-mediated complement activation is functionally competent, since it inserts into the membranes of neuronal precursor cells and renders them permeable to small molecules.

Thus, Aβ directly and independently activates the ACP as well as the CCP, leading to the formation of covalent Aβ-C3b and Aβ-iC3b complexes; generates C5a; and mediates assembly of functionally active C5b-9 complexes in vitro. These findings have potential implications for understanding the mechanisms which lead to continuing neuronal damage and altered glial functions in the vicinity of NP, and thus to the progression of AD. First, they provide an explanation for the association of bound C3 with Aβ in NP (1-3), since covalently bound C3b molecules in NP would remain bound and provide a nidus for chronic complement activation. Second, C5a generated by Aβ-mediated complement activation could be responsible for the increased numbers of activated astrocytes and microglia

around NP compared with diffuse Aβ plaques (28), since these cells possess C5a receptors and are activated and migrate in response to C5a (6, 29-31). C5a could also trigger the release of proinflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) from glial cells, as it does from other cell types (26, 32); proinflammatory cytokines are increased in the AD brain (2, 28, 33). These cytokines could further activate glial cells and alter neuronal and glial functions (28, 32). Third, incoming activated glial cells could bind and remain adherent, via their complement receptors, to C3 activation fragments attached to Aβ (6). Fourth, C5b-9 insertion into cell membranes provides an explanation for the association of this complex with dystrophic neurites in NP (2, 3). Although not likely to be directly cytotoxic for neurons, since they bear CD59 (6, 34), C5b-9 as well as C5b-7 and C5b-8 complexes could alter neuronal functional properties over time by chronic low-level triggering of various cellular signaling pathways (35). If this inflammation-based scenario is verified, complement inhibitors should be evaluated for use in AD. Such inhibitors would need to pass the blood-brain barrier, target both complement activation pathways, and prevent C5b-9 activation.

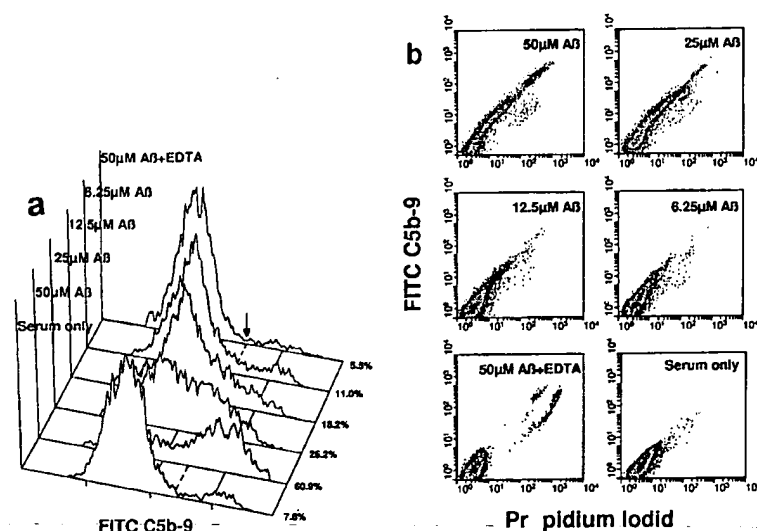


Figure 4. C5b-9 generated by Aβ 1-42-mediated complement activation is functionally active. (a) NT2 cells were incubated with preaggregated Aβ 1-42 at the designated concentrations in NHS, or in NHS containing 10 mM EDTA; serum only lacks Aβ. Flow cytometric analyses with a rabbit Ab to C5b-9 neoantigens are shown. Numbers (right), Percentage of C5b-9+ cells, as determined by their relationship to the marker (arrow, dashed line). (b) Density plot analyses of propidium iodide and C5b-9 (FITC C5b-9) reactivities are shown. For clarity, only live cells are depicted.

We thank T. Hugli, D. Isenman, J. Rogers, and S. Webster for helpful discussions; G. Nemerow for PB50, and Athena Neurosciences, Inc. (South San Francisco, CA), for 10D5. We also thank Todd S. Bixby for his expert technical assistance.

This work was supported by National Institutes of Health grant NS-34682, and grant SFP-1141 from Novartis.

Address correspondence to Neil R. Cooper, Department of Immunology, IMM-19, 10550 North Torrey Pines Rd., La Jolla, CA 92037. Phone: 619-784-8152; Fax: 619-784-8472; E-mail: nrcooper@scripps.edu

Received for publication 30 December 1997 and in revised form 27 May 1998.

References

1. Eikelenboom, P., and F.C. Stam. 1982. Immunoglobulins and complement factors in senile plaques. *Acta Neuropathol.* 57:239-242.
2. McGeer, P.L., and E.G. McGeer. 1995. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res. Brain Res. Rev.* 21:195-218.
3. Rogers, J., N.R. Cooper, S. Webster, J. Schultz, P.L. McGeer, S.D. Styren, W.H. Civin, L. Brachova, B. Bradt, P. Ward, and I. Lieberburg. 1992. Complement activation by β -amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci. USA.* 89:10016-10020.
4. Müller-Eberhard, H.J. 1988. Molecular organization and function of the complement system. *Annu. Rev. Biochem.* 57:321-347.
5. Cooper, N.R. 1998. Biology of the complement system. In *Inflammation: Basic Principles and Clinical Correlates*. J. Gallin and R. Snyderman, editors. Lippincott-Raven Publishers, Philadelphia. In press.
6. Morgan, B.P., and P. Gasque. 1996. Expression of complement in the brain: role in health and disease. *Immunol. Today.* 17:461-466.
7. Jiang, H., D. Burdick, C.G. Glabe, C.W. Cotman, and A.J. Tenner. 1994. β -Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. *J. Immunol.* 152:5050-5059.
8. Chen, S., R.C.A. Frederickson, and K.R. Brunden. 1996. Neuroglial-mediated immunoinflammatory responses in Alzheimer's disease: complement activation and therapeutic approaches. *Neurobiol. Aging.* 17:781-787.
9. Webster, S., B. Bradt, J. Rogers, and N.R. Cooper. 1997. Aggregation state-dependent activation of the classical complement pathway by the amyloid β peptide (A β). *J. Neurochem.* 69:388-398.
10. Cadman, E.D., and P.S. Puttfarcken. 1997. β -Amyloid peptides initiate the complement cascade without producing a comparable effect on the terminal pathway *in vitro*. *Exp. Neurol.* 146:388-394.
11. Webster, S., B. Bonnell, and J. Rogers. 1997. Charge-based binding of complement component C1q to the Alzheimer amyloid β -peptide. *Am. J. Pathol.* 150:1531-1536.
12. Velazquez, P., D.H. Cribbs, T.L. Poulos, and A.J. Tenner. 1997. Aspartate residue 7 in amyloid β -protein is critical for classical complement pathway activation: implications for Alzheimer's disease pathogenesis. *Nat. Med.* 3:77-79.
13. Schreiber, R.D., M.K. Pangburn, P.H. Lesavre, and H.J. Müller-Eberhard. 1978. Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. *Proc. Natl. Acad. Sci. USA.* 75:3948-3952.
14. Wickham, T.J., P. Mathias, D.A. Cheres, and G.R. Nemerow. 1993. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. *Cell.* 73:309-319.
15. Mayer, M.M. 1961. Complement and complement fixation. In *Experimental Immunochimistry*. E.A. Kabat and M.M. Mayer, editors. Charles C. Thomas Publisher, Springfield, IL. 133-241.
16. Wagner, J.L., and T.E. Hugli. 1984. Radioimmunoassay for anaphylatoxins: a sensitive method for determining complement activation products in biological fluids. *Anal. Biochem.* 136:75-88.
17. McGeer, P.L., H. Akiyama, S. Itagaki, and E.G. McGeer. 1989. Immune system response in Alzheimer's disease. *Can. J. Neurol. Sci.* 16:516-527.
18. McGeer, P.L., H. Akiyama, S. Itagaki, and E.G. McGeer. 1989. Activation of the classical complement pathway in brain tissue of Alzheimer patients. *Neurosci. Lett.* 107:341-346.
19. Law, S.K.A., N.A. Lichtenberg, and R.P. Levine. 1979. Evidence for an ester linkage between the labile binding site of C3b and receptive surfaces. *J. Immunol.* 123:1388-1394.
20. Gadd, K.J., and K.B.M. Reid. 1981. The binding of complement component C3 to antibody-antigen aggregates after activation of the alternative pathway in human serum. *Biochem. J.* 195:471-480.
21. Miller, G.W., and V. Nussenzweig. 1975. A new complement function: solubilization of antigen-antibody aggregates. *Proc. Natl. Acad. Sci. USA.* 72:418-422.
22. Fujita, T., Y. Takata, and N. Tamura. 1981. Solubilization of immune precipitates by six isolated alternative pathway proteins. *J. Exp. Med.* 154:1743-1751.
23. Harris, M.E., K. Hensley, D.A. Butterfield, R.A. Leedle, and J.M. Carney. 1995. Direct evidence of oxidative injury produced by the Alzheimer's β -amyloid peptide (1-40) in cultured hippocampal neurons. *Exp. Neurol.* 131:193-202.
24. Dyrks, T., E. Dyrks, T. Hartmann, C. Masters, and K. Beyreuther. 1992. Amyloidogenicity of β A4 and β A4-bearing amyloid protein precursor fragments by metal-catalyzed oxidation. *J. Biol. Chem.* 267:18210-18217.
25. Hensley, K., D.A. Butterfield, N. Hall, P. Cole, R. Subramanian, R. Mark, M.P. Mattson, W.R. Markesbery, M.E. Harris, M. Aksenov, et al. 1996. Reactive oxygen species as causal agents in the neurotoxicity of the Alzheimer's disease-associated amyloid beta peptide. *Ann. NY Acad. Sci.* 786:120-134.
26. Hugli, T.E. 1984. Structure and function of the anaphylatoxins. *Springer Semin. Immunopathol.* 7:193-219.

27. Shen, Y., J.A. Halperin, and C.-M. Lee. 1995. Complement-mediated neurotoxicity is regulated by homologous restriction. *Brain Res.* 671:282-292.
28. Griffin, W.S.T., J.G. Sheng, G.W. Roberts, and R.E. Mrak. 1995. Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. *J. Neuropathol. Exp. Neurol.* 54:276-281.
29. Yao, J., L. Harvath, D.L. Gilbert, and C.A. Colton. 1990. Chemotaxis by CNS macrophage, the microglia. *J. Neurosci. Res.* 27:36-42.
30. Lacy, M., J. Jones, S.R. Whittemore, D.L. Haviland, R.A. Wetsel, and S.R. Barnum. 1995. Expression of the receptors for the C5a anaphylatoxin, interleukin-8 and FMLP by human astrocytes and microglia. *J. Neuroimmunol.* 61:71-78.
31. Ilshner, S., C. Nolte, and H. Kettenmann. 1996. Complement factor C5a and epidermal growth factor trigger the activation of outward potassium currents in cultured murine microglia. *Neuroscience.* 73:1109-1120.
32. Benveniste, E.N. 1992. Inflammatory cytokines within the central nervous system: sources, function, and mechanism of action. *Am. J. Physiol.* 263:C1-C16.
33. Selkoe, D.J. 1994. Alzheimer's disease: a central role for amyloid. *J. Neuropathol. Exp. Neurol.* 53:438-447.
34. Vedeler, C., E. Ulvestad, L. Bjorge, G. Conti, K. Williams, S. Mork, and R. Matre. 1994. The expression of CD59 in normal human nervous tissue. *Immunology.* 82:542-547.
35. Rus, H.G., F. Niculescu, and M.L. Shin. 1996. Sublytic complement attack induces cell cycle in oligodendrocytes. *J. Immunol.* 156:4892-4900.

Complement activation by β -amyloid in Alzheimer disease

JOSEPH ROGERS*[†], NEIL R. COOPER[‡], SCOTT WEBSTER*, JAMES SCHULTZ*, PATRICK L. McGEER[§],
SCOT D. STYREN*, W. HAROLD CIVIN*, LIBUSE BRACHOVA*, BONNIE BRADT[‡], PAMELA WARD[¶],
AND IVAN LIEBERBURG[¶]

*L. J. Roberts Center, Sun Health Research Institute, 13220 North 105th Avenue, Sun City, AZ 85351; [†]Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037; [‡]Kinsmen Laboratory for Neurological Research, University of British Columbia Medical School, Vancouver, BC V6T 1Z3, Canada; and [§]Athena Neurosciences, Inc., 800F Gateway Boulevard, South San Francisco, CA 94080

Communicated by Susan E. Leeman, June 23, 1992

ABSTRACT Alzheimer disease (AD) is characterized by excessive deposition of the β -amyloid peptide (β -AP) in the central nervous system. Although several lines of evidence suggest that β -AP is neurotoxic, a mechanism for β -AP toxicity in AD brain remains unclear. In this paper we provide both direct *in vitro* evidence that β -AP can bind and activate the classical complement cytolytic pathway in the absence of antibody and indirect *in situ* evidence that such actions occur in the AD brain in association with areas of AD pathology.

Alzheimer disease (AD) is characterized by excessive central nervous system (CNS) deposition of the β -amyloid peptide (β -AP), a 40- to 42-amino acid peptide derived from a larger amyloid precursor protein (APP) (1–3). Although no specific mechanism of β -AP deposition has yet been formally proven, there are several lines of evidence (4–6) that, once generated, β -AP causes direct or indirect toxicity to CNS neurons. Proposed mechanisms of AD neurotoxicity include membrane changes (7), alterations in Ca^{2+} homeostasis (6, 8), excitotoxicity (5, 6), and disruption of cytoskeletal or axon transport systems (9, 10). However, no single AD pathogenic mechanism has yet achieved a wide consensus of acceptance.

In addition to studies of β -AP, over the last decade a number of investigators have noted that the AD brain exhibits many of the classical markers of immune-mediated damage. These include elevated numbers of major histocompatibility complex class I- and II-immunoreactive microglia (cells believed to be an endogenous CNS form of the peripheral macrophage) (11–15) and astrocytes expressing interleukin 1 (16) and α_1 -antichymotrypsin (17) (both acute phase reactants). Of particular importance, complement proteins of the classical pathway have been immunohistochemically detected in the AD brain (12, 13, 18–20), and we have noted that they most often appear associated with β -AP-containing pathological structures such as senile plaques. Proteins specific to the alternative pathway do not appear to be present (12, 13, 18). The first step in the classical complement pathway entails binding of the C1q component of C1, with subsequent activation of the C1r and C1s components. This is followed by a complex series of autocatalytic reactions, proceeding through C4, C2, and C3, and culminating in formation of the membrane attack complex (MAC), C5b–9. The MAC inserts a lytic plug in adjacent cell membranes, mediating cellular toxicity (21). Although C1q binding to the Fc region of immunoglobulins is the most common mechanism for initiating the classical pathway, several substrates—including viruses, parasites, and mannan-binding protein—have also been demonstrated to activate C1 and to do so in an antibody-independent fashion (22). In this paper we present six converging lines of evidence suggesting that β -AP

activates the classical pathway complement cascade without mediation by immunoglobulin. This previously unrecognized mechanism may contribute significantly to the neurotoxicity of β -AP as well as to the pathophysiology of neuronal dysfunction characteristic of AD.

MATERIALS AND METHODS

Human Brain Samples. Brain materials were obtained at autopsy from volunteer AD patients and nondemented elderly (ND) controls through the Sun Health Research Institute Tissue Donation Program. Postmortem intervals were well matched and did not exceed 3.5 hr in any case. Samples of the superior frontal gyrus, hippocampal complex, amygdala, brain stem, and cerebellum were dissected as approximately 1-cm³ blocks, postfixed in ice-cold 4% (wt/vol) paraformaldehyde (0.1 M sodium phosphate buffer, pH 7.4) for 16–24 hr, cryoprotected in 2% dimethyl sulfoxide/10% glycerol (vol/vol) followed by 2% dimethyl sulfoxide/20% glycerol (in 0.1 M phosphate buffer, pH 7.4) for 48 hr each, sectioned at 20 or 40 μ m on a freezing microtome, and stored at –20°C in a cryopreservation solution composed of 33% glycerol, 33% polyethylene glycol, and 33% 0.1 M phosphate buffer, pH 7.4 (vol/vol).

Immunohistochemistry. Sections were removed from glycol storage and washed six times for 15 min each in TBS (0.01 M Tris-HCl, pH 7.6/0.09 M NaCl). Endogenous peroxidase was blocked by incubation for 5 min with 0.3% H_2O_2 /50% methanol (vol/vol) in TBS, followed by three 15-min rinses in TBS/0.05% Triton X-100. We then blocked for 1 hr in 3% bovine serum albumin (BSA) in TBS/0.05% Triton X-100. Although it does not materially affect the results to block with normal serum, we did not do so because of the possibility that normal serum might add exogenous complement proteins, contaminating anti-complement immunohistochemistry. Sections were incubated overnight with primary antibody at 4°C with gentle agitation, washed three times for 15 min each with TBS/0.05% Triton X-100, incubated for 1 hr with 1:50 biotin-conjugated secondary antibody (Vector Laboratories), and allowed to react with diaminobenzidine (DAB) as in standard ABC/DAB immunohistochemistry (Vector Laboratories). Primary and secondary antibodies were diluted in TBS/0.7% λ carrageenan/0.5% Triton X-100/0.2% sodium azide. For double-label immunohistochemistry, sections were next washed three times for 15 min each in TBS, incubated overnight with the second primary antibody at 4°C (gentle agitation), washed three times for 15 min each in TBS/0.05% Triton X-100, incubated for 30 min with second-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AD, Alzheimer disease; β -AP, β -amyloid peptide; APP, amyloid precursor protein; APPs 751, C-terminal truncated, secreted form of the 751-residue APP; CNS, central nervous system; MAC, membrane attack complex; ND, nondemented elderly; BSA, bovine serum albumin; TGF, transforming growth factor.

[†]To whom reprint requests should be addressed.

ary antibody (Vector Laboratories), and allowed to react as in standard ABC-alkaline phosphatase (AP) immunohistochemistry (Vector Laboratories), including a levamisole blocking step. Finally, the sections were counterstained with thioflavin S and mounted. Preabsorption with appropriate antigens (when available), deletion of primary antibody, and absence of staining in ND patients were always employed as negative controls.

Dot Blots for C1q Binding. Samples (200 μ l) of 5 μ M β -AP fragments β -AP-(1-38) and β -AP-(1-28), the 751-residue APP secreted form (APP_S 751) (11), or various control peptides [here, BSA and the transmembrane domain of transforming growth factor (TGF)] in TBS were blotted onto Westran polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell). The membrane was rinsed in TBS, blocked for 1 hr in TBS plus 5% nonfat powdered milk, washed, and incubated with human C1q (Quidel) at 10 μ g/ml for 2 hr. The membrane was then washed, incubated with rabbit antibodies to human C1q (DAKO, Carpinteria, CA) diluted 1:1000 in TBS plus 5% nonfat powdered milk overnight at 4°C, washed in TBS, incubated in biotinylated goat antibodies to rabbit IgG (Vector Laboratories) at a dilution of 1:50 in TBS plus 5% nonfat powdered milk for 2 hr, washed, and processed according to the ABC/DAB method (Vector Laboratories). All washes were five times for 5 min each, and all incubations and washes were performed with gentle agitation. C1q was deleted on adjacent blots to control for nonspecific immunoreactivity of peptides with the primary and secondary antibodies.

CH₅₀ Assay for Complement Activation. The CH₅₀ assay is a standard complement activation assay that has been widely used for over a decade. The assay performed here is thoroughly described by Mayer (23). Test solutions containing β -AP-(1-38) added to normal serum at 125, 250, and 500 μ g/ml were employed. The results with β -AP-(1-38) were referenced to the normal serum vehicle without β -AP-(1-38).

ELISA for Complement Activation. Samples (100 μ l each, all 5.0 μ M) of BSA, TGF (transmembrane domain), APP_S 751 (24), β -AP-(1-38), β -AP-(1-28), β -AP-(1-16), β -AP-(17-28), β -AP-(24-35), and β -AP-(10-28) were plated in a 96-well ELISA plate, blocked with 1% BSA/1% powdered milk in 10 mM sodium phosphate, pH 7.4/0.9 mM NaCl (PBS), washed once with PBS/1% BSA, and incubated 40 min at 37°C with 50 μ l of fresh normal human serum diluted 1:20 in PBS. Wells were then washed 10 times with PBS/1% BSA, incubated 1 hr at 37°C with 100 μ l of a mouse monoclonal anti-C3b antibody (Quidel, San Diego) at 300 ng/ml, washed 10 times with PBS, and incubated 30 min at 37°C with horseradish peroxidase-conjugated goat antibodies to mouse IgG (Chemicon). To each well, 100 μ l 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS)/peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added at room temperature. Optical densities (ODs) of wells at 405 nm were recorded at 2, 6, and 8 min. ODs of wells from which the test peptides had been deleted were subtracted to control for nonspecific background reactivity. Wells from which the primary antibody had been deleted gave only background measures.

RESULTS

C1q Immunoreactivity Colocalizes with β -AP-Containing AD Pathological Structures. Fig. 1 shows typical immunoreactivity for a rabbit antiserum directed against human C1q in AD and ND superior frontal gyrus. In ND patients, no specific staining is observed (Fig. 1A), whereas under the same conditions profuse labeling of numerous large roughly spherical structures is revealed in AD samples (Fig. 1B). Thioflavin counterstaining (Fig. 1B *Insert*) demonstrates that these C1q immunoreactive structures are senile plaques, a

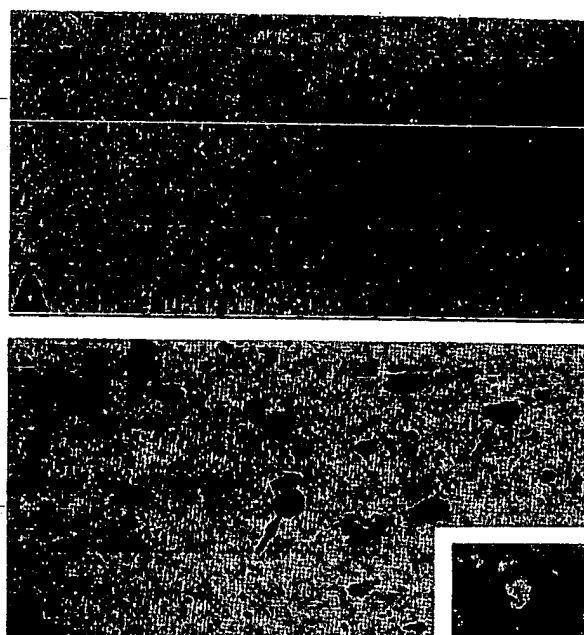


FIG. 1. Typical immunoreactivity in superior frontal gyrus of clinically and neuropathologically confirmed ND (A) and AD (B) patients when a rabbit antiserum directed against human C1q (1:1000 dilution) was used. In AD, staining is almost exclusively limited to profiles with the morphology and distribution of senile plaques (arrows), as well as some neurofibrillary tangles. Virtually all thioflavin-positive plaques are positive for C1q immunoreaction product (*Insert*) (fluorescence plus dim bright-field optics). (Calibration bar equals 50 μ m.)

pathological hallmark of AD associated with β -AP deposits. Some thioflavin-positive neurofibrillary tangles also colocalize with C1q, as has been previously reported (12, 13, 20). These results are representative, without exception, of findings in 20 AD and 10 ND patients. Moreover, C1q colocalization with sites of β -AP-containing structures or β -AP immunoreactivity is equally evident in such brain areas as temporal cortex, amygdala, and hippocampus, a result that has been replicated, without exception, in 20 AD patients.

C1q Immunoreactivity Does Not Colocalize with Immunoglobulin Immunoreactivity. Although immunoglobulin has been reported to occur in AD brain tissue (13, 18, 19, 25), it does not colocalize with C1q immunohistochemically (Fig. 2). Rather, immunoglobulin immunoreactivity occurs on scattered clusters of neurons not associated with senile plaques (Fig. 2A), and C1q immunoreactivity occurs in the context of β -AP-containing structures such as senile plaques (Figs. 1B and 2B). These findings have been repeated in 15 AD patients with seven different anti-immunoglobulin antibodies or Fab fragments (e.g., 1:500 sheep anti-human IgG Fc from Bioproducts for Science, Indianapolis; 1:500 sheep anti-human IgG Fab from Bioproducts for Science; 1:2000 goat F(ab')₂ anti-human IgG from Cappel Laboratories). The fact that immunoreactive immunoglobulin can be detected at all in these studies is important, since it shows that the failure to observe immunoglobulin in association with C1q is not the result of inadequate techniques. Much of the immunoglobulin staining in brain has been suggested to be an artifact due to postmortem vascular changes (26). Our studies tend to confirm this hypothesis. Immunoglobulin immunoreactivity similar to that of the representative AD patient in Fig. 2B is equally apparent in nondemented young adult patients (S.D.S. and J.R., unpublished data). The staining is patchy throughout all areas of brain, tending to occur diffusely in

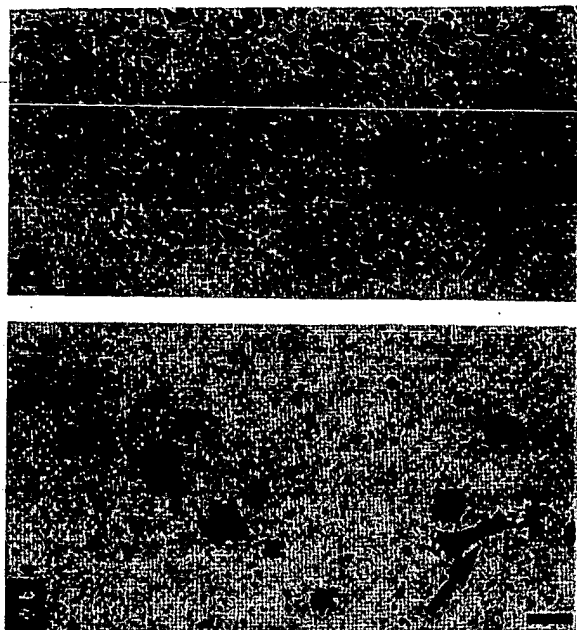


FIG. 2. Serial sections of AD anterior cingulate gyrus that have reacted with a polyclonal antiserum directed against human immunoglobulin (A) or a monoclonal antibody directed against C1q (B). Immunoglobulin and C1q immunoreactivities do not colocalize here or in other similar sections. For orientation, arrows indicate a blood vessel in the two adjacent sections. (Calibration bar equals 60 μ m.)

clusters several hundred μ m in diameter that are centered on or near large blood vessels. By contrast, C1q immunoreactivity is evident only in areas of brain that feature significant β -AP deposition, such as association cortex and limbic system, and colocalization is to sites of β -AP deposition or β -AP immunoreactivity, not necessarily to blood vessels. It is therefore most parsimonious to conclude that the localization of C1q to β -AP-containing AD pathological sites is antibody independent.

β -AP Binds C1q *in Vitro*. Dot blots of immobilized β -AP-(1-38), β -AP-(1-28), and APP_S 751 appear to bind physiological concentrations of C1q *in vitro* (Fig. 3). The result with APP_S 751 is interesting since this peptide contains β -AP-(1-15) as its carboxyl terminus. Similar results have been obtained in four different replicate experiments in the laboratory of J.R., as well as in experiments performed in the laboratory of P.L.M.

β -AP Activates the Classical Complement Pathway in CH₅₀ Assays. Colocalization of complement proteins with β -AP-containing AD structures does not necessarily prove complement activation nor does it provide a mechanism for such activation. To test for the latter, a standard *in vitro* assay of complement activation, CH₅₀, was employed (23). Referenced to the percent activity of normal serum, the CH₅₀ remaining at β -AP-(1-38) concentrations of 125, 250, and 500 μ g/ml was 85.0%, 45.8%, and 0.0%, respectively. This antibody-independent complement activation is approximately 10-fold greater than that for equivalent amounts of the oncornaviruses rat leukemia virus, primate Rauscher leukemia virus, Rauscher leukemia virus, and Moloney leukemia virus under similar assay conditions (22). These results have been confirmed in three different replicate experiments.

β -AP Activates the Classical Complement Pathway in ELISAs. The CH₅₀ data were confirmed and extended by using an ELISA for measurement of complement activation. This assay is based on detection of newly formed C3b after incubation of test peptides with normal serum. Results are

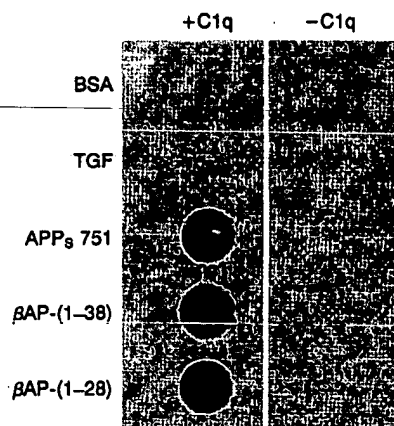


FIG. 3. C1q binding to BSA, the transmembrane domain of TGF, APP_S 751, β -AP-(1-38), and β -AP-(1-28). C1q was deleted from the right-lane-to-control for nonspecific reactivity of the C1q antibody and detection system. Untreated membranes and control peptides—here, BSA and TGF—do not show detectable C1q binding, whereas peptides containing, as a minimum, the β -AP-(1-15) sequence—here, APP_S 751, β -AP-(1-38), and β -AP-(1-28)—do. C1q was obtained from Quidel and BSA (fraction V) was obtained from Sigma. APP_S 751, β -AP fragments, and TGF were the generous gift of Athena Neurosciences (San Francisco).

shown in Fig. 4. Essentially background measures of complement activation were observed with BSA, TGF (transmembrane domain), and APP_S 751, whereas activation by β -AP-(1-38) and β -AP-(1-28) was as much as 4- to 5-fold higher. Interestingly, β -AP fragments containing all or part of the first 16 β -AP amino acid residues [e.g., β -AP-(1-28), -(1-16), -(10-28)] activated complement, whereas β -AP fragments not containing these residues [e.g., β -AP-(17-28), -(24-35)] did not. Interactions with the alternative pathway are unlikely to explain these results: (i) alternative pathway activation is weak to absent at the 1:20 serum dilutions used in the present experiments; and (ii) activation is abolished when MgEGTA-treated serum is used—a condition wherein alternative pathway activation can occur, but classical pathway activation cannot. These data were replicated over three different assays. Measurement of ODs at time points other than those shown in Fig. 4 gave lower or higher values depending on length of incubation, but the pattern of results remained the same. In summary, these data show that β -AP and β -AP fragments containing all or part of residues 1-16 can functionally activate the classical pathway complement cascade without mediation by immunoglobulin.

β -AP Activates the Full Classical Pathway Complement Cascade *in Vivo*. Like that for C1q, immunoreactivity for other complement proteins occurs in the context of β -AP-containing AD pathological structures (Fig. 5). For example, serial sections through plaques stained by using antibodies directed against C1q and C4d coupled with thioflavin histochemistry reveal that C1q and C4d immunoreactivities colocalize with thioflavin-positive plaques (Fig. 5 A-C). Because attachment of C4d requires full activation of the C1 complex (8), these *in situ* data strongly support the *in vitro* observation that β -AP functionally activates complement. Similar results can be shown for the final steps of complement activation, including the MAC, C5b-9 (Fig. 5 D and E), indicating that β -AP not only activates complement but may lead to complement-mediated pathogenesis at sites of β -AP deposition.

DISCUSSION

The converging evidence presented here strongly supports the hypothesis that β -AP can directly activate complement in

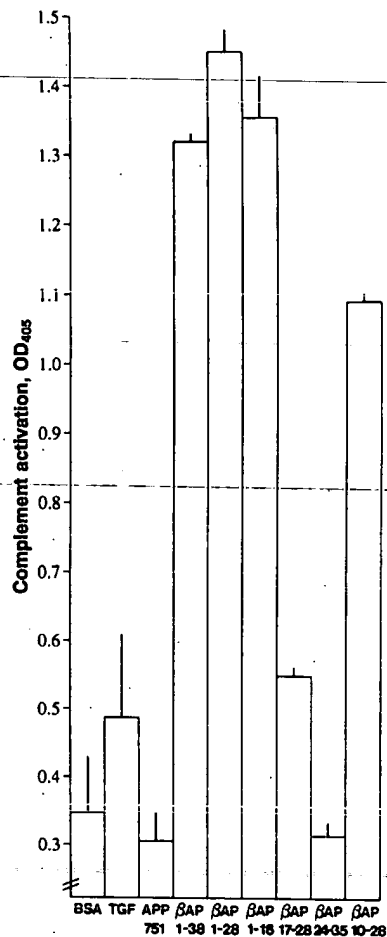


FIG. 4. Complement activation by β -AP and related peptides in an ELISA. Mean ODs (\pm SEM) at 6 min, reflecting formation of the activated C3 fragment C3b, are shown.

the AD brain without the usual mediation of immunoglobulin. We have demonstrated the mechanism for this process *in vitro*, and we have shown that the process may occur *in situ* in the context of β -AP deposits and β -AP-containing AD pathological structures. Colocalization of C5b-9, the MAC, with β -AP immunoreactivity is particularly important in this regard, since the formation of the MAC is direct evidence that full-blown complement activation has occurred at the site.

Although it remains possible that β -AP-mediated complement activation in AD is simply a response to pathogenesis rather than a cause, several points argue for a more primary role. In the periphery, complement-mediated membrane attack can be a generally beneficial mechanism for removing infected or damaged cells, even if some healthy cells are removed in the process by bystander lysis (21). Nerve cells, however, are postmitotic. For this reason, membrane attack on nervous tissues adjacent to β -AP deposits would almost certainly have deleterious consequences, even as a purely undirected bystander phenomenon. In addition, other laboratories have now demonstrated that complement defense mechanisms such as the membrane inhibitor of reactive lysis (MIRL, CD59) (14), vitronectin (S-protein) (27), and SP40,40 (28) are up-regulated in AD brain. The need for these mechanisms also supports the idea that β -AP-mediated complement activation has pathogenetic significance in AD. Finally, the presence of the MAC, C5b-9, generated as the result of complement activation, implies binding to brain tissue (healthy as well as dystrophic), since the β -AP deposits at the

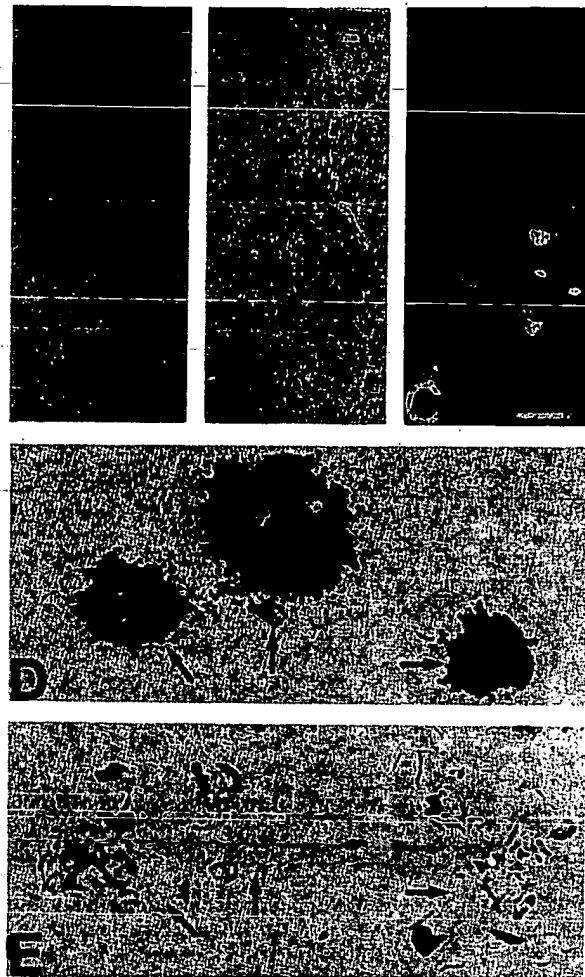


FIG. 5. (A-C) Serial 50- μ m-thick sections from AD superior frontal gyrus, showing immunohistochemical colocalization of C1q (A), C4d (B), and thioflavin S-positive senile plaques (C). The full range of complement proteins colocalize within the same plaques. D and E, for example, illustrate C4d and C5b-9 immunoreactivities, respectively, in serial sections from AD hippocampus. Note that although the same plaques are labeled, different elements within the plaques are stained. This is the expected result, since C5b-9 (but not C4d) requires a cell membrane point of attachment. Mouse monoclonal antibodies to human C4d and C5b-9 were obtained from Quidel and used at 1:1000 dilutions. (Calibration bar equals 100 μ m in A-C and 50 μ m in D and E).

site do not offer an appropriate cell membrane target. This cytolytic process, together with other sequelae of complement activation such as opsonization (targeting) of tissue for scavenger cell attack and anaphylotoxin signaling to scavenger cells, is difficult to construe as other than pathogenic.

Antibody-independent complement activation has ample precedent both as a basic immunological phenomenon and as a mechanism of human disease (21, 22). In the fluid phase, C1q binding to naturally occurring nonimmunoglobulin substrates is typically handled by such regulatory factors as C1 inhibitor, so that full-blown complement activation does not occur (21). For this reason, APP_S 751 would be unlikely to activate complement *in vivo* (and does not do so *in vitro*). Likewise, as a cellular or membrane-bound protein (1), APP would still be unlikely to activate complement because cellular defense mechanisms such as decay-accelerating factor, homologous restriction factor (HRF), and membrane cofactor protein (MCP) would come into play (21), and, as noted

above, several of these have been recently demonstrated to be up-regulated in AD.

β -AP deposits, on the other hand, would have no such protection from the complement cascade. Our experiments show that β -AP and β -AP fragments actively bind C1q, the first component of the C1 complex (Fig. 3). Insoluble in the fluid phase (1–3), β -AP would be subject to this C1q binding without subsequent regulation by fluid-phase inhibitors. As an insoluble self-aggregating deposit (1–3), β -AP itself would also not be able to generate the cellular regulatory factors that inhibit complement activation. Thus, the antibody-independent β -AP-mediated complement activation that we have demonstrated *in vitro* would be likely to run its full course *in vivo*, having an impact on immediately adjacent tissues—a point that is strongly reinforced by colocalization of the MAC, the final component of complement activation, with β -AP deposits, C1q, and C4d (Figs. 1 and 5).

Several important markers of immune function have now been demonstrated in AD brain tissue, many of them highly colocalized with AD pathological structures. These include immunoreactive markers for major histocompatibility complex class I and II glycoproteins, cytokines, Fc and complement receptors, complement regulatory factors, and classical pathway complement proteins from C1q through the MAC (11–15, 18–20, 27, 28). Recent data also suggest that mRNA for complement proteins may exist in CNS cells (29), raising the possibility of endogenous complement production within the CNS. For these and other reasons, several investigators have suggested that classical pathway complement activation occurs in AD brain (12, 13, 18, 19), but no demonstration of this process has been provided beyond the level of immunohistochemical visualization of complement, nor has any mechanism for complement activation been proffered. Our demonstration of β -AP-induced complement activation provides such a mechanism and may have explanatory power for other facets of AD pathogenesis. For example, the antibody-independent nature of β -AP-induced complement activation may help explain why classical pathway complement proteins are so easily demonstrated in the AD brain (12, 13, 18–20), but the expected co-occurrence of AD-specific antibodies has been difficult to detect in the great majority of cases (18, 26).

The cause of the dementia of AD is neuronal and neuritic damage, but the cause of AD neuronal and neuritic damage has remained elusive. The fact that we use excessive deposition of β -AP as a unique hallmark and diagnostic criterion may underlie and perhaps justify the experimental attention this peptide has received as a pathogenic factor in AD. However, our laboratory and others (30) have noted extensive β -AP deposition in postmortem brain samples from several elderly patients who were considered to be nondemented throughout life. Likewise, the cerebellum typically exhibits little if any clinical or neural pathology in AD but contains significant numbers of diffuse β -AP deposits (31). These findings suggest that β -AP may be a necessary but not sufficient element in AD pathogenesis, a concept that fits well with the present data and other published reports. For example, full complement activation appears to occur in the context of compacted association cortex β -AP deposits, but it does not occur in the context of diffuse cerebellar β -AP deposits (32, 33). Although both sites contain β -AP (albeit in different aggregation states), it is the association cortex that exhibits concurrent complement activation and neuritic pathology. We also note that AD may well have multiple pathogenic bases and, indeed, this could extend even to the β -AP molecule itself. For example, β -AP might have an innate but limited neurotoxicity (4, 5) that is exacerbated by complement activation. Such hypotheses may be amenable to testing by using neuronal culture and other models.

This work was supported by grants from the U.S. National Institute on Aging (J.R.).

1. Selkoe, D. J. (1991) *Neuron* 6, 487–492.
2. Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., & Müller-Hill, B. (1987) *Nature (London)* 325, 733–736.
3. Glenner, G. G. & Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* 120, 885–890.
4. Yankner, B. A., Duffy, L. K., & Kirschner, D. A. (1990) *Science* 250, 279–282.
5. Koh, J. Y., Yang, L. L., & Cotman, C. W. (1990) *Brain Res.* 533, 315–321.
6. Mattson, M. P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I., & Rydel, R. E. (1992) *J. Neurosci.* 12, 376–389.
7. Nitsch, R. M., Blusztajn, J. K., Pittas, A. G., Slack, B. E., Growdon, J. H., & Wurtman, R. J. (1992) *Proc. Natl. Acad. Sci. USA* 89, 1671–1675.
8. Gibson, G. E. & Peterson, C. (1987) *Neurobiol. Aging* 8, 329–343.
9. Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., & Binder, L. I. (1986) *Proc. Natl. Acad. Sci. USA* 83, 4913–4917.
10. Kowall, N. W. & Kosik, K. S. (1987) *Ann. Neurol.* 22, 639–643.
11. McGeer, P. L., Akiyama, H., Itagaki, S., & McGeer, E. G. (1989) *Neurosci. Lett.* 107, 341–344.
12. McGeer, P. L., Akiyama, H., Itagaki, S., & McGeer, E. G. (1989) *Can. J. Neurol. Sci.* 16, 516–524.
13. Rogers, J. & McGeer, P. L. (1992) in *Alzheimer's Disease*, eds. Khachaturian, Z., & Blass, J. (Dekker, New York), pp. 147–163.
14. McGeer, P. L., Walker, D. G., & Akiyama, H. (1991) *Brain Res.* 544, 315–320.
15. Styren, S. D., Civin, W. H., & Rogers, J. (1990) *Exp. Neurol.* 110, 93–107.
16. Griffin, W. S. T., Stanley, L. C., Linc, C., White, L., MacLeod, V., Perrot, L. J., White, C. L., & Araoz, C. (1989) *Proc. Natl. Acad. Sci. USA* 86, 7611–7615.
17. Abraham, C. R., Selkoe, D. J., & Potter, H. (1988) *Cell* 52, 487–501.
18. Eikelenboom, P. & Stam, F. C. (1982) *Acta Neuropathol.* 57, 239–244.
19. Ishii, T., Haga, S., & Kametari, F. (1988) in *Immunology and Alzheimer's Disease*, eds. Pouplard-Barthelais, A., Emile, J., & Christen, Y. (Springer, Berlin), pp. 17–29.
20. Rogers, J., Brachova, L., Webster, S., Lue, L.-F., & Schultz, J., *Res. Immunol.*, in press.
21. Cooper, N. R. (1987) in *Basic and Clinical Immunology*, eds. Stites, D. P., & Terr, A. I. (Appleton & Lange, Norwalk, CT), 6th Ed., pp. 114–127.
22. Cooper, N. R. (1985) *Adv. Immunol.* 37, 151–171.
23. Mayer, M. M. (1961) in *Experimental Immunochimistry*, eds. Kabat, E. A., & Mayer, M. M. (Thomas, Springfield, IL), 2nd Ed., pp. 133–140.
24. Oltersdorf, T., Ward, P. J., Henriksson, T., Beattie, E. C., Neve, R., Lieberburg, I., & Fritz, L. C. (1990) *J. Biol. Chem.* 265, 4492–4500.
25. Gaskin, F., Kingsley, B. S., & Fu, S. M. (1987) *J. Exp. Med.* 165, 245–250.
26. Mori, S., Sternberger, N. H., Herman, M. M., & Sternberger, L. A. (1991) *Lab. Invest.* 64, 345–351.
27. Akiyama, H., Kawamata, T., Dedhar, S., & McGeer, P. L. (1991) *J. Neuroimmunol.* 32, 19–28.
28. May, P. C., Lampert-Etchells, M., Johnson, S. A., Poirier, J., Masters, J. N., & Finch, C. E. (1990) *Neuron* 5, 831–838.
29. Walker, D. G., & McGeer, P. L. (1992) *Mol. Brain Res.* 14, 109–116.
30. Katzman, R., Terry, R., DeTeresa, R., Brown, T., Davies, P., Fuld, P., Renbing, X., & Peck, A. (1988) *Ann. Neurol.* 23, 138–144.
31. Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., & Nakazato, Y. (1989) *Acta Neuropathol.* 77, 314–319.
32. Lue, L., & Rogers, J. (1992) *Neurosci. Abs.* 18, 733.
33. Rozemuller, J., Stam, F., & Eikelenboom, P. (1990) *Neurosci. Lett.* 119, 75–78.



Proceedings of the National Academy of Sciences, Vol 89, 10016-10020,
Copyright © 1992 by National Academy of Sciences

ARTICLE

Complement Activation by β -Amyloid in Alzheimer Disease

J Rogers, NR Cooper, S Webster, J Schultz, PL McGeer, SD Styren, WH Civin, L Brachova, B Bradt, P Ward and I Lieberburg

Alzheimer disease (AD) is characterized by excessive deposition of the β -amyloid peptide (β -AP) in the central nervous system. Although several lines of evidence suggest that β -AP is neurotoxic, a mechanism for β -AP toxicity in AD brain remains unclear. In this paper we provide both direct in vitro evidence that β -AP can bind and activate the classical complement cytolytic pathway in the absence of antibody and indirect in situ evidence that such actions occur in the AD brain in association with areas of AD pathology.

- ▶ [Reprint \(PDF\) Version of this Article](#)
- ▶ Similar articles found in:
 [PNAS Online](#)
 [PubMed](#)
- ▶ [PubMed Citation](#)
- ▶ Search Medline for articles by:
 [Rogers, J.](#) || [Lieberburg, I.](#)
- ▶ Alert me when:
 [new articles cite this article](#)
- ▶ [Download to Citation Manager](#)

This article has been cited by other articles:

- Budayova-Spano, M., Lacroix, M., Thielens, N. M., Arlaud, G. J., Fontecilla-Camps, J. C., Gaboriaud, C. (2002). The crystal structure of the zymogen catalytic domain of complement protease C1r reveals that a disruptive mechanical stress is required to trigger activation of the C1 complex. *EMBO J.* 21: 231-239 [[Abstract](#)] [[Full Text](#)]
- Tacnet-Delorme, P., Chevallier, S., Arlaud, G. J. (2001). β -Amyloid Fibrils Activate the C1 Complex of Complement Under Physiological Conditions: Evidence for a Binding Site for A β on the C1q Globular Regions. *The JI* 167: 6374-6381 [[Abstract](#)] [[Full Text](#)]
- Soane, L., Cho, H.-J., Niculescu, F., Rus, H., Shin, M. L. (2001). C5b-9 Terminal Complement Complex Protects Oligodendrocytes from Death by Regulating Bad Through Phosphatidylinositol 3-Kinase/Akt Pathway. *The JI* 167: 2305-2311 [[Abstract](#)] [[Full Text](#)]
- Webster, S. D., Galvan, M. D., Ferran, E., Garzon-Rodriguez, W., Glabe, C. G., Tenner, A. J. (2001). Antibody-Mediated Phagocytosis of the Amyloid β -Peptide in Microglia Is Differentially Modulated by C1q. *The JI* 166: 7496-7503 [[Abstract](#)] [[Full Text](#)]
- Selkoe, D. J. (2001). Alzheimer's Disease: Genes, Proteins, and Therapy. *Physiol. Rev* 81: 741-766 [[Abstract](#)] [[Full Text](#)]
- Speth, C., Stöckl, G., Mohsenipour, I., Würzner, R., Stoiber, H., Lass-Flörl, C., Dierich, M. P. (2001). Human Immunodeficiency Virus Type 1 Induces Expression of Complement Factors in Human Astrocytes. *J. Virol.* 75: 2604-2615 [[Abstract](#)] [[Full Text](#)]
- Yang, L.-B., Li, R., Meri, S., Rogers, J., Shen, Y. (2000). Deficiency of Complement Defense Protein CD59 May Contribute to Neurodegeneration in Alzheimer's Disease. *J. Neurosci.* 20:

7505-7509 [[Abstract](#)] [[Full Text](#)]

- Nepomuceno, R. R., Tenner, A. J. (1998). C1qRP, the C1q Receptor That Enhances Phagocytosis, Is Detected Specifically in Human Cells of Myeloid Lineage, Endothelial Cells, and Platelets. *The JJ* 160: 1929-1935 [[Abstract](#)] [[Full Text](#)]

- Sahu, A., Soulika, A. M., Morikis, D., Spruce, L., Moore, W. T., Lambris, J. D. (2000). Binding Kinetics, Structure-Activity Relationship, and Biotransformation of the Complement Inhibitor Compstatin. *The JJ* 165: 2491-2499 [[Abstract](#)] [[Full Text](#)]
- Singhrao, S. K., Neal, J. W., Rushmere, N. K., Morgan, B. P., Gasque, P. (2000). Spontaneous Classical Pathway Activation and Deficiency of Membrane Regulators Render Human Neurons Susceptible to Complement Lysis. *Am J Pathol* 157: 905-918 [[Abstract](#)] [[Full Text](#)]
- Dodel, R., Du, Y., Bales, K. R., Gao, F., Paul, S. (1999). Sodium Salicylate and 17{beta}-Estradiol Attenuate Nuclear Transcription Factor NF- κ B Translocation in Cultured Rat Astroglial Cultures Following Exposure to Amyloid A{beta} 1-40 and Lipopolysaccharides. *J Neurochem* 73: 1453-1460 [[Abstract](#)] [[Full Text](#)]

- Sayah, S., Ischenko, A. M., Zhakhov, A., Bonnard, A.-S., Fontaine, M. (1999). Expression of Cytokines by Human Astrocytomas Following Stimulation by C3a and C5a Anaphylatoxins: Specific Increase in Interleukin-6 mRNA Expression. *J Neurochem* 72: 2426-2436 [[Abstract](#)] [[Full Text](#)]
- Gaboriaud, C., Rossi, V., Bally, I., Arlaud, G. J., Fontecilla-Camps, J. C. (2000). Crystal structure of the catalytic domain of human complement C1s: a serine protease with a handle. *EMBO J.* 19: 1755-1765 [[Abstract](#)] [[Full Text](#)]
- Farkas, I., Baranyi, L., Takahashi, M., Fukuda, A., Liposits, Zs., Yamamoto, T., Okada, H. (1998). A neuronal C5a receptor and an associated apoptotic signal transduction pathway. *J Physiol Lond* 507: 679-687 [[Abstract](#)] [[Full Text](#)]
- Stoltzner, S. E., Grenfell, T. J., Mori, C., Wisniewski, K. E., Wisniewski, T. M., Selkoe, D. J., Lemere, C. A. (2000). Temporal Accrual of Complement Proteins in Amyloid Plaques in Down's Syndrome with Alzheimer's Disease. *Am J Pathol* 156: 489-499 [[Abstract](#)] [[Full Text](#)]
- Wagner, S. L., Munoz, B. (1999). Modulation of amyloid {beta} protein precursor processing as a means of retarding progression of Alzheimer's disease. *J. Clin. Invest.* 104: 1329-1332 [[Full Text](#)]
- González-Scarano, F., Baltuch, G. (1999). MICROGLIA AS MEDIATORS OF INFLAMMATORY AND DEGENERATIVE DISEASES. *Annu. Rev. Neurosci.* 22: 219-240 [[Abstract](#)] [[Full Text](#)]
- Price, D. L., Tanzi, R. E., Borchelt, D. R., Sisodia, S. S. (1998). ALZHEIMER'S DISEASE: Genetic Studies and Transgenic Models. *Annu. Rev. Genet.* 32: 461-493 [[Abstract](#)] [[Full Text](#)]
- Yasojima, K., Schwab, C., McGeer, E. G., McGeer, P. L. (1999). Up-Regulated Production and Activation of the Complement System in Alzheimer's Disease Brain. *Am J Pathol* 154: 927-936 [[Abstract](#)] [[Full Text](#)]
- Rogers, J. T., Leiter, L. M., McPhee, J., Cahill, C. M., Zhan, S.-S., Potter, H., Nilsson, L. N. G. (1999). Translation of the Alzheimer Amyloid Precursor Protein mRNA Is Up-regulated by Interleukin-1 through 5'-Untranslated Region Sequences. *J. Biol. Chem.* 274: 6421-6431 [[Abstract](#)] [[Full Text](#)]
- Blasko, I., Marx, F., Steiner, E., Hartmann, T., Grubeck-loebenstein, B. (1999). TNF{alpha} plus IFN{gamma} induce the production of Alzheimer β -amyloid peptides and decrease the secretion of APPs. *FASEB J.* 13: 63-68 [[Abstract](#)] [[Full Text](#)]
- Stohr, J., Schindler, G., Rothe, G., Schmitz, G. (1998). Enhanced Upregulation of the Fc{gamma} Receptor IIIa (CD16a) During In Vitro Differentiation of ApoE4/4 Monocytes. *Arterioscler Thromb* 18: 1424-1432 [[Abstract](#)] [[Full Text](#)]
- Bradt, B. M., Kolb, W. P., Cooper, N. R. (1998). Complement-dependent Proinflammatory Properties of the Alzheimer's Disease beta -Peptide. *J. Exp. Med.* 188: 431-438 [[Abstract](#)] [[Full Text](#)]

- Makrides, S. C. (1998). Therapeutic Inhibition of the Complement System. *Pharmacological Reviews* 50: 59-88 [[Abstract](#)] [[Full Text](#)]
- Lezoualc'h, F., Sagara, Y., Holsboer, F., Behl, C. (1998). High Constitutive NF-kappa B Activity Mediates Resistance to Oxidative Stress in Neuronal Cells. *J. Neurosci.* 18: 3224-3232 [[Abstract](#)] [[Full Text](#)]
- Kuo, Y.-M., Emmerling, M. R., Vigo-Pelfrey, C., Kasunic, T. C., Kirkpatrick, J. B., Murdoch, G. H., Ball, M. J., Roher, A. E. (1996). Water-soluble Abeta(N-40, N-42) Oligomers in Normal and Alzheimer Disease Brains. *J. Biol. Chem.* 271: 4077-4081 [[Abstract](#)] [[Full Text](#)]
- Johnson-Wood, K., Lee, M., Motter, R., Hu, K., Gordon, G., Barbour, R., Khan, K., Gordon, M., Tan, H., Games, D., Lieberburg, I., Schenk, D., Seubert, P., McConlogue, L. (1997). Amyloid precursor protein processing and Abeta 42 deposition in a transgenic mouse model of Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* 94: 1550-1555 [[Abstract](#)] [[Full Text](#)]
- Möller, T., Nolte, C., Burger, R., Verkhratsky, A., Kettenmann, H. (1997). Mechanisms of C5a and C3a Complement Fragment-Induced [Ca²⁺]_i Signaling in Mouse Microglia. *J. Neurosci.* 17: 615-624 [[Abstract](#)] [[Full Text](#)]
- Selkoe, D. J. (1996). Amyloid beta -Protein and the Genetics of Alzheimer's Disease. *J. Biol. Chem.* 271: 18295-18298 [[Full Text](#)]
- Garzon-Rodriguez, W., Sepulveda-Becerra, M., Milton, S., Glabe, C. G. (1997). Soluble Amyloid Abeta -(1-40) Exists as a Stable Dimer at Low Concentrations. *J. Biol. Chem.* 272: 21037-21044 [[Abstract](#)] [[Full Text](#)]
- Paresce, D. M., Chung, H., Maxfield, F. R. (1997). Slow Degradation of Aggregates of the Alzheimer's Disease Amyloid beta -Protein by Microglial Cells. *J. Biol. Chem.* 272: 29390-29397 [[Abstract](#)] [[Full Text](#)]
- Giulian, D., Haverkamp, L. J., Yu, J. H., Karshin, W., Tom, D., Li, J., Kirkpatrick, J., Kuo, Y.-M., Roher, A. E. (1996). Specific Domains of beta -Amyloid from Alzheimer Plaque Elicit Neuron Killing in Human Microglia. *J. Neurosci.* 16: 6021-6037 [[Abstract](#)] [[Full Text](#)]

[HOME](#) [HELP](#) [FEEDBACK](#) [SUBSCRIPTIONS](#) [ARCHIVE](#) [SEARCH](#) [TABLE OF CONTENTS](#)

Copyright © 1992 by the National Academy of Sciences